39727

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of

Capon et al.

Serial No.: 08/238,405

Filed: 5 May 1994

For: CHIMERIC CHAINS FOR RECEPTOR-ASSOCIATED SIGNAL TRANSDUCTION PATHWAYS

Group Art Unit: 1647

Examiner: Hayes, R.

REFERENCES CITED IN APPEAL BRIEF

BOX AF
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Attached hereto please find copies, in triplicate, of U.S. Pat. Nos. 5,712,149; 6,103,521; 5,837,544; and 5,504,000 as referred to on page 16 of the Appellants' Brief on Appeal that was submitted on 26 March 2002.

Respectfully submitted,

Dean H. Nakamura
Attorney for Applicants
Reg. No. 33,981

Roylance, Abrams, Berdo
& Goodman L.L.P.
1300 19th Street, N.W., Suite 600
Washington, D.C. 20036-2680
(202) 659-9076

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United States Patent [19]

Roberts

[11] Patent Number: 5,712,149

[45] Date of Patent: Jan. 27, 1998

[54] **CHIMERIC RECEPTOR MOLECULES FOR DELIVERY OF CO-STIMULATORY SIGNALS**

[75] Inventor: **Margo R. Roberts**, San Francisco, Calif.

[73] Assignee: **Cell Genesys, Inc.**, Foster City, Calif.

[21] Appl. No.: **383,749**

[22] Filed: **Feb. 3, 1995**

[51] **Int. Cl.**[6] **C07K 14/705**; C07K 19/00; C12N 15/62

[52] **U.S. Cl.** **435/252.3**; 435/69.7; 435/320.1; 530/350; 536/23.4

[58] **Field of Search** 435/64.7, 252.3, 435/320.1; 530/350; 536/23.4

[56] **References Cited**

PUBLICATIONS

Irving et al, *Cell* 64:841–901, 08 Mar. 1991.

Romeo et al., *Cell* 68:889–897, 06 Mar. 1992.

Romeo et al, *Cell* 64:1037–1046, 08 Mar. 1991.

Letourneur et al, *P.N.A.S.* 88:8405–8409, Oct. 1991.

Eslla–et al, *P.N.A.S.* 90:720–724, Jan. 1993.

Romeo et al., "Activation of . . . Intracellular Domains", Cold Spring Harbor Symposia on Quantitative Biology, vol. 57, 117 (1992).

Linsley et al., "The Role of the CD28 Receptor During T Cell Responses to Antigen,", *Annu. Rev. Immounol.* 11:191–212 (1993).

June et al., "The B7 and CD28 receptor families," *Immunology Today* 15(7):321–331 (1994).

Schwartz, R.H., "Costimulation of T Lymphocytes:The Role of CD28, CTLA–4, and B7/BB1 in Interleukin–2 Production and Immunotherapy," *Cell*71:1055–1068 (1992).

Stein et al., "The Cytoplasmic Domain of CD28 Is both Necessary and Sufficient for Costimulation of Interleukin–2 Secretion and Association with Phosphatidlinositol 3'–Kinase," *Mol. and Cell. Biol.*14(5):3392–3402 (1994).

Harding et al., "CD28–B7 Interactions Allow the Induction of CD8+Cytotoxic T Lymphocytes in the Absence of Exogenous Help," *J. Exp. Med.* 177:1791–1796 (1993).

Riddell et al. "The use of anti–CD3 and anti–CD28 monoclonal antibodies to clone and expand human anatigen–specific T cells," *J. of Immun. Methods*128:189–201 (1990).

Chen et al., "Tumor Immunogenicity Determines the Effect of B7 Costimulation on T Cell–mediated Tumor Immunity," *J. Exp. Med.*179:523–532 (1994).

Chen et al., "Costimulation of Antitumor Immunity by the B7 Counterreceptor for the T Lymphocyte Molecules CD28 and CTLA–4," *Cell*71:1093–1102 (1992).

Baskar et al., "Constitutive expression of B7 restores immuogenicity of tumor cells expressing trncated major hisocompatibility complex class II molecules, "*PNAS*90:5687–5690 (1993).

He et al., "A Role in Transmembrane Signaling for the Cytoplasmic Domain of the CD2 Lymphocyte Surface Antigen," *Cell*54:979–984 (1988).

Bierer et al., "Synergistic T Cell Activation via the Physiological Ligands for CD2 and the T Cell Receptor," *J. Exp. Med.*168:1145–1156 (1988).

Roberts et al., "Targeting of Human Immunodeficiency Virus–Infected Cells by CD8+T Lymphocytes Armed with Universal T–Cell Receptors," *Blood*84(9):2878–2889 (1994).

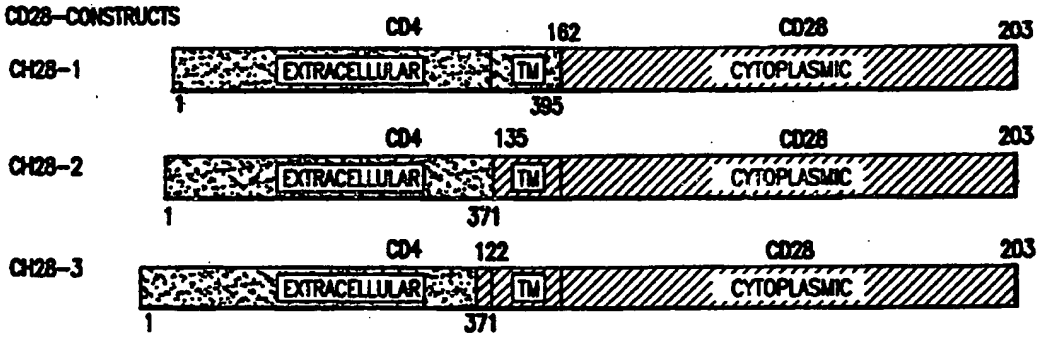
Primary Examiner—John Ulm

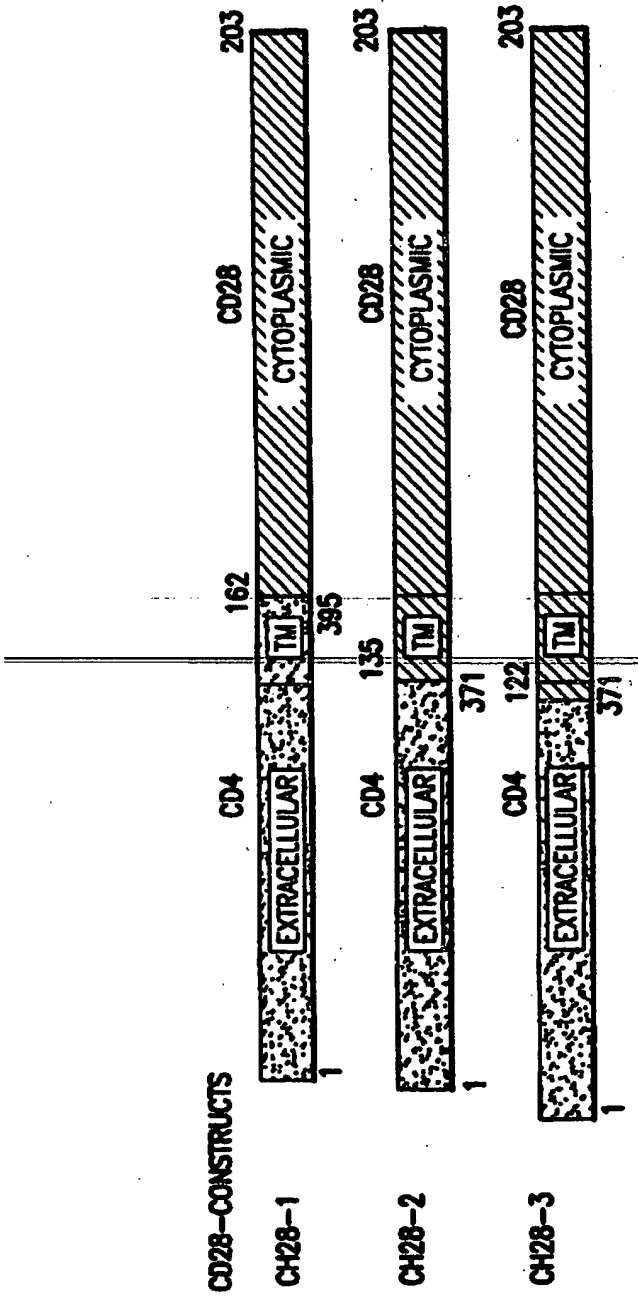
Attorney, Agent, or Firm—Sughrue,Mion,Zinn,Macpeak & Seas, PLLC

[57] **ABSTRACT**

The present invention is directed to novel chimeric co-stimulatory receptor proteins and DNA sequences encoding these proteins. The chimeric receptors comprise at least three domains in a single chain molecule: an extracellular ligand binding domain, a transmembrane domain and a cytoplasmic co-stimulatory effector function signaling domain that acts synergistically with an effector function signal in the host cell. Novel hybrid co-stimulatory receptor proteins include a second cytoplasmic effector function signaling domain. The invention further relates to expression cassettes containing the nucleic acids encoding the novel chimeric receptors, to host cells expressing the novel chimeric receptors and to methods of using the receptors to co-stimulate effector functions in the cells and for using cells expressing the receptors for treatment of cancer, disease and viral infections.

25 Claims, 3 Drawing Sheets

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CH28-2
CH28-3
CD4
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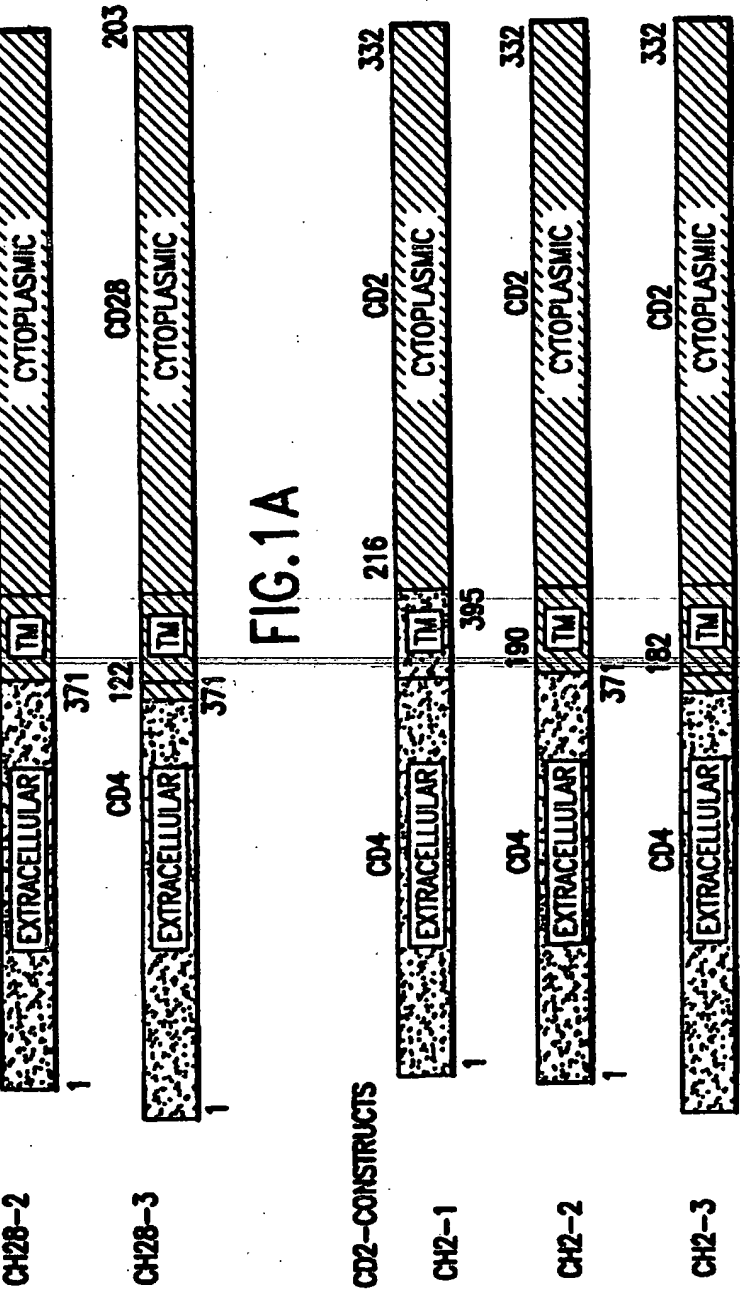
TM
CD28
CYTOPLASMIC
1
162
203
135
122
371
395

FIG. 1A


CD2-CONSTRUCTS
CH2-1
CH2-2
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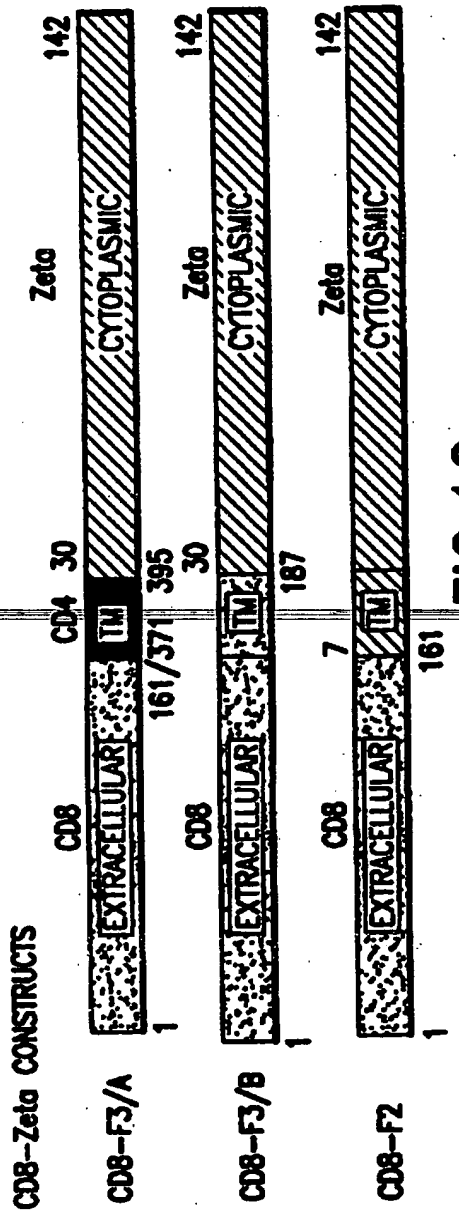
190
182
371
395

FIG. 1B


CD8-Zeta CONSTRUCTS
CD8-F3/A
CD8-F3/B
CD8-F2
CD8
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Zeta
EXTRACELLULAR
TM
CYTOPLASMIC
1
161/371
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187
7
161
142

FIG.1C

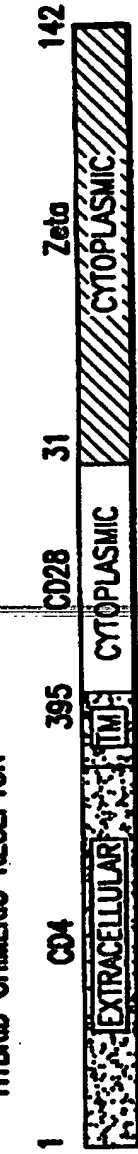
HYBRID CHIMERIC RECEPTOR
CD4
CD28
Zeta
EXTRACELLULAR
TM
CYTOPLASMIC
1
395
162
31
203
142

FIG.1D

1.	CGTATTGGATCCGAGGAAACCAACCCCTAAG	(SEQ ID NO:1)
2.	AATATTGGGCCCGGCAGAAATCCACAGTGC	(SEQ ID NO:2)
3.	AATATTGGCGCCCCTAGCCCATCGTCAGGA	(SEQ ID NO:3)
4.	AATATTGGATCCGGCTTCTGGATAGGCGTC	(SEQ ID NO:4)
5.	CACCACCAGCACCCAAAATGGCTGCACCGGGGTGGA	(SEQ ID NO:5)
6.	TAGGGGACTTGGACAAAGTGGCTGCACCGGGGTGGA	(SEQ ID NO:6)
7.	CCTCTGTTTTTTCCTTTTGACACAGAAGAAGATGCC	(SEQ ID NO:7)
8.	GCCAATGATGAGATAGATTGGCTGCACCGGGGTGGA	(SEQ ID NO:8)
9.	ACCTTTCTCTGGACAGCTTGGCTGCACCGGGGTGGA	(SEQ ID NO:9)
10.	CCTGCTCCTCTTACTCCTCCGGCACCTGACACAGAA	(SEQ ID NO:10)
11.	CCTGCTCCTCTTACTCCTGAAGAAGATGCCTAGCCC	(SEQ ID NO:11)
12.	AATATTGAATTCCGAGCTTCGAGCCAA	(SEQ ID NO:12)
13.	AATATTGGTTACCAGTGGCTGTTGCACAGGG	(SEQ ID NO:13)
14.	CGCCCCCCAGCACAATCAGGGCCATTGCGCCCCCCG-CCGCCGCTGGCCGGCAC	(SEQ ID NO:14)
15.	CTGCGCTCCTGCTGAACTTCACTCTATTTGCAAACA-CGTCTTCGGTTCCT	(SEQ ID NO:15)
16.	CATCCAGCAGGTAGCAGAGTTTGGGTGCGCCCCCCG-CCGCTGGCCGGCAC	(SEQ ID NO:16)

FIG. 2

CHIMERIC RECEPTOR MOLECULES FOR DELIVERY OF CO-STIMULATORY SIGNALS

TECHNICAL FIELD

The field of this invention relates to novel chimeric surface membrane proteins for use in co-stimulatory signal transduction.

BACKGROUND

Regulation of cell activities is frequently achieved by the binding of a ligand to a surface membrane receptor comprising an extracellular and a cytoplasmic domain. The formation of the complex between the ligand and the extracellular portion of the receptor results in a conformational change in the cytoplasmic portion of the receptor which results in a signal transduced within the cell. In some instances, the change in the cytoplasmic portion results in binding to other proteins, where other proteins are activated and may carry out various functions. In some situations, the cytoplasmic portion is autophosphorylated or phosphorylated, resulting in a change in its activity. These events are frequently coupled with secondary messengers, such as calcium, cyclic adenosine monophosphate, inositol phosphate, diacylglycerol, and the like. The binding of the ligand to the surface membrane receptor results in a particular signal being transduced.

Engagement of the TCR alone is not sufficient to induce activation of resting naive or memory T cells. Full, productive T cell activation requires a second co-stimulatory signal from a competent antigen-presenting cell (APC). Co-stimulation is achieved naturally by the interaction of the co-stimulatory cell surface receptor on the T cell with the appropriate counter-receptor on the surface of the APC. An APC is normally a cell of host origin which displays a moiety which will cause the stimulation of an immune response. APCs include monocyte/macrophages, dendritic cells, B cells, and any number of virally-infected or tumor cells which express a protein on their surface recognized by T cells. To be immunogenic APCs must also express on their surface a co-stimulatory molecule. Such APCs are capable of stimulating T cell proliferation, inducing cytokine production, and acting as targets for cytolytic T cells upon direct interaction with the T cell. (Linsley and Ledbetter, *Ann. Rev. Immunol.* 4:191–212 (1993); Johnson and Jenkins, *Life Sciences* 55:1767–1780 (1994); June et al., *Immunol. Today* 15:321–331 (1994); and Mondino and Jenkins, *J. Leuk. Biol.* 55:805–815 (1994)).

There are a number of situations in which the immune system fails to respond properly because the APCs lack the counter-receptor molecules necessary for co-stimulation. The result is an immune system which is paralyzed in response to that particular moiety.

For T lymphocytes in particular, induction of effector functions requires two biochemically distinct signals delivered through engagement of unique cell surface membrane receptors, usually one delivered through the T cell's specific antigen receptor (TCR) and the other via a so-called co-stimulatory receptor. Engagement of the co-stimulatory molecule together with the TCR is necessary for optimal levels of IL-2 production, proliferation and clonal expansion, and generation of effector functions such as the production of immunoregulatory cytokines, induction of antibody responses from B cells, and induction of cytolytic activity. More importantly, engagement of the TCR in the absence of the co-stimulatory signal results in a state of non-responsiveness, called anergy. Anergic cells fail to become activated upon subsequent stimulation through the TCR, even in the presence of co-stimulation, and in some cases may be induced to die by a programmed self-destruct mechanism.

In certain situations, for example where APCs lack the counter-receptor molecules necessary for co-stimulation, it would be beneficial to have the co-stimulatory signal induced by virtue of employing a ligand other than the natural ligand for the co-stimulatory receptor. This might be, for example, the same ligand as that recognized by the TCR (i.e., the same moiety, such that if one signal is received, both signals will be received), or another cell surface molecule known to be present on the target cells (APCs).

The primary co-stimulatory receptor is the CD28 molecule on T cells which interacts with a so-called "counter-receptor" ligand called B7 on APCs. Co-stimulation is achieved naturally by the interaction of the CD28 surface membrane receptor on T cells with the B7 counter-receptor on APCs.

CD28 is a homodimer of 44 kD subunits, a member of the immunoglobulin (Ig) gene superfamily. CD28 is expressed on thymocytes and the majority of mature T cells in peripheral lymphoid tissues. it is expressed on virtually all CD4+ T cells and approximately 50% of CD8+ T cells. The subpopulation of CD8 T cells expressing CD28 contains the precursors for cytotoxic effector cells, while the reciprocal population has the potential to act as suppressor cells (Linsley et al., 1993 supra).

The biochemical mechanism of CD28 signal transduction has not been clearly elucidated but it is clear that (i) CD28 stimulation alone is not sufficient to activate cells, and (ii) the signal mediated by CD28 must be different from the CD3/TCR signal because the CD28 signal synergizes with the CD3/TCR signal. A detailed summary of CD28 signal transduction can be found in June et al., 1994, supra, and Linsley and Ledbetter, 1993, supra. A hallmark of CD28 signaling is that it is at least partially resistant to CsA, and therefore must have a component which is independent to the calcium-dependent phosphatase calcineurin. This is in direct contrast to CD3/TCR signaling which is completely inhibited by CsA. CD28 signaling involves the activation of phosphoinositide (PI) 3-kinase. CD28 ligation at the cell surface results in phosphorylation of Tyr 191 in the cytoplasmic domain of CD28. This phosphorylation event in turn drives the association of CD28 with PI 2-kinase via the -Y-M-N-T-P-R-amino acid sequence motif in the cytoplasmic domain of CD28 and the SH3 domain of PI 3-kinase. The phosphorylation of CD28 at Tyr 191 is thought to be an important regulator of CD28 signaling. Activation of PI 3-kinase activates an undefined cascade of second messengers which complete the CD28 signal.

CD28 co-stimulation results in maximum IL-2 production and enhances secretion of several other immunoregulatory cytokines, in particular those associated with TH1 cells. Enhanced secretion of cytokines occurs by two mechanisms: (i) the stabilization of cytokine mRNA (Mondino and Jenkins (1994), supra) and (ii) an increase in the rate of transcription via a CD28 response element in the promoters of the affected cytokines (Thompson et al., *Proc. Natl. Acad. Sci. USA* 86:1333–1337 (1989); Fraser and Weiss, *Mol. Cell. Biol.* 12:4357–4363 (1992); and Fraser et al., *Science* 251:313–316 (1991)). Cytokine production by both CD4 and CD8 T cells has been reported to be enhanced. Among the cytokines whose production is increased by CD28 co-stimulation are: IL-2, γ-IFN, TNF-α, lymphotoxin, and

GM-CSF (Thompson et al., supra; June et al., supra; Chen et al., *J. Exp. Med.* 179:523–532 (1994); Kuiper et al., *Immunology* 83:38–44 (1994); and Mondino and Jenkins, supra). Of importance is the possibility that CD28 co-stimulation could render CD8 cells capable of sufficient autocrine IL-2 production to be independent of exogenous "help" (Harding and Allison, *J. Exp. Med.* 177:1791–1796 (1993)) from CD4+ T cells for example, and/or trigger alternative signal transduction pathways which promote IL-2 independent proliferation (Riddell and Greenberg, *J. Immunol. Methods* 128:189–201 (1990)).

CD28 co-stimulation also results in an increase in the rate of cell division, although this may be secondary to the increase in IL-2 production. Furthermore, direct increases in thymidine incorporation in vitro are only seen at suboptimal concentrations of anti-CD3 (Thompson et al., supra). This increase in the rate of cell division can be translated to an increased cloning efficiency of antigen-specific T cell lines in vitro (Riddell and Greenberg, supra).

In contrast, lack of CD28 co-stimulation at the time of antigen encounter (or CD3/TCR stimulation) results in anergy, a state of specific un-responsiveness. Antibodies to B7 or soluble ligands such as CTLA-Ig (Linsley et al. *J. Exp. Med.* 174:561–569 (1991)) have been used in vitro and in vivo to block antigen-specific immune responses and prevent graft rejection (Thompson et al., supra; Fraser and Weiss, supra; and Fraser et al., supra) by interfering with the interaction between B7 and CD28 (Johnson and Jenkins, supra; Schwartz, R. H., *Cell* 71:1065–1068 (1992); Linsley and Ledbetter, supra). Antibodies to CD28 can substitute for co-stimulation by APCs in inducing immune responses and protecting T cells from anergy (Johnson and Jenkins, supra, and Linsley and Ledbetter, supra).

In certain tumor models, in vitro and in vivo, lack of expression of co-stimulatory molecules by tumor cells correlates with the ability to evade immunological destruction. This effect can be reversed by engineering the tumor cells to express B7 as shown by treatment of experimental tumors in animals and in vitro T cell responses to tumor cells transduced with B7 (Chen et al., *Cell* 71:1093–1102 (1992); Schwartz et al., supra; Jung et al., *PNAS* 84:4611–4615 (1987); Guo et al., *Science* 263:518–520 (1994); Chen et al., *Cancer Res.* 54:5420–5423 (1994); Hodge et al., *Cancer Res.* 54:5552–5555 (1994); Li et al., *J. Immunol.* 153:421–428 (1994); Townsend and Allison, *Science* 259:368-(1993); Booker et al., *PNAS* 90:5687–5690 (1993); Chen et al., *J. Exp. Med.* 179:523–532 (1994); and Harding and Allison (1993), supra).

Decreased CD28 expression in both CD4 and CD8 T cell populations from HIV-infected individuals correlates with defects in T cell function, tendency to undergo activation-induced apoptosis, and disease progression. Correction of defects in T cell function and protection from apoptosis (a programmed cell death mechanism initiated by aberrant signal transduction) is observed in vitro when cells are cultured with anti-CD28 antibodies (Brinchman et al., *J. Inf. Dis.* 169:730–738 (1994); Caruso et al., *Scand. J. Immunol.* 40:485–490 (1994); Landay et al., *Clin. Immunol. Immunopathol.* 69:106–116 (1993); Gougeon et al., *Science* 260:1269–1270 (1993); Groux et al., *J. Exp. Med.* 175:331–340 (1992); Meyaard et al., *Science* 257:217–219 (1992); and Choremi-Papadopoulou et al., *J. Aids* 7:245–253 (1994)).

A second receptor, CTLA-4, bears significant structural homology to CD28 and interacts with the same B7 family counter-receptors as CD28 (Linsley and Ledbetter, supra; June et al., supra, and Mondino and Jenkins, supra). CTLA-4 was identified in a cDNA library made from murine cytolytic T lymphocytes (Linsley and Ledbetter, supra). CTLA-4 and CD28 share the same intron and exon organization and are genetically linked on human chromosome 2. Like CD28, CTLA-4 has a putative PI 3-kinase interaction motif in its cytoplasmic tail. CTLA-4 expression is induced in activated T cells (expression is restricted to the CD28+ populations), and is expressed in lower amounts on the cell surface than CD28, but has a much higher affinity for the B7 family counter-receptors. The level of homology between CTLA-4 and CD28 suggests that CTLA-4 signaling proceeds via a biochemical pathway similar to that of CD28 and that it performs a complementary signaling role to CD 28.

Another receptor molecule, CD21, found on virtually all thymocytes and T cells, has also been shown to synergize with CD3/TCR stimulation to augment T cell activation (Bierer et al., *J. Exp. Med.* 168:1145 (1988)). CD2 is a 50 kd single chain surface membrane glycoprotein with two immunoglobulin-like extracellular domains (Williams, *Immunol. Today* 8:298–303 (1987)), a transmembrane domain, and a cytoplasmic domain which is involved in signal transduction (He et al., *Cell* 54:979–985 (1988)). In contrast to CD28 and CTLA-4, which alone are unable to activate T cells, the use of monoclonal antibodies (anti-$T11_1$ and anti-$T11_2$) which bind to distinct epitopes on CD 2, together, induce T cell proliferation and cytokine production (Meuer et al., *Cell* 36:897 (1984)). However, activation of T cells via CD2 is dependent on the expression in the cell of the zeta chain of CD3 (Howard et al., *J. Exp. Med.* 176:139–145 (1992)).

In addition to CD28, CTLA-4, CD2, several other surface receptors have been reported to provide co-stimulation for T cell activation through the CD3/TCR. These include, for example, CD5, ICAM-1, LFA-1 (CD11a/CD18) (Ledbetter et al., *J. Immunol.* 135:2331 (1985); Damle et al., *J. Immunol.* 148:1985–1992; Mondino and Jenkins, *J. Leuk. Biol.* 55:805–815 (1994)). The signaling pathways utilized by these co-stimulatory molecules share the common property of acting in synergy with the primary T cell receptor activation signal.

The production of chimeric effector function receptor proteins which initiate signaling in a cell that results in activation of a second messenger pathway in response to an inducer binding to the extracellular portion of these receptors is the subject of U.S. Pat. No. 5,359,046, the disclosure of which is incorporated in its entirety herein. These chimeric effector function receptor proteins comprise three domains in a single protein moiety, namely an extracellular ligand binding domain, a cytoplasmic effector function signaling domain and a transmembrane domain linking the extracellular and cytoplasmic domain together. The cytoplasmic domain and extracellular domain are not naturally associated. These chimeric effector function receptors can transduce a signal in their host cells in response to the binding of different ligands, in a non-MHC restricted fashion, to the extracellular domain. These receptors are useful for directing the activity of cells expressing the receptors for a particular effector functional.

A co-stimulatory chimeric receptor in which an extracellular ligand binding domain is linked to the signal transducing domain of a co-stimulatory molecule, such as CD28, could have as its target virtually any cell surface moiety of interest. Using such chimeric receptors, the ligand which provides co-stimulation may be selected to support a desired immune response where for some reason the natural ligand is missing or less useful. The co-stimulatory receptor could

provide a signal that is synergistic with the primary effector activation signal, i.e. the TCR signal or the chimeric effector function receptor signal, and can complete the requirements for activation under conditions where stimulation of the TCR or chimeric effector function receptor is suboptimal and might otherwise be detrimental to the function of the cell. These receptors can support immune responses, particularly of T cells, by permitting the use of ligands other than the natural ligand to provide the required co-stimulatory signal.

SUMMARY OF THE INVENTION

Novel co-stimulatory receptor chimeric DNA sequences, expression cassettes and vectors containing these sequences, as well as cells containing the chimeric DNA and novel chimeric receptor proteins expressed from the sequences, are provided where the novel co-stimulatory chimeric DNA sequences comprise three domains which do not naturally exist together: (1) at least one cytoplasmic domain, which normally transduces a co-stimulatory signal resulting in activation of a messenger system, (2) at least one transmembrane domain, which crosses the outer cellular membrane, and (3) at least one extracellular receptor domain which serves to bind to a ligand and transmit a signal to the cytoplasmic domain, resulting in a co-stimulatory signal in the host cell in which the chimeric DNA is expressed. Particularly, cytoplasmic DNA sequences of co-stimulatory molecules such as the CD28, CTLA-4 or CD2 cell surface receptors are employed joined to other than their natural extracellular domain by a transmembrane domain. In this manner, host cells that express the chimeric co-stimulatory receptor protein can receive the necessary co-stimulatory signal by contact with the ligand as contrasted with the normal mode of activation of the cytoplasmic domain. Additional embodiments of the co-stimulatory receptors include hybrid chimeric receptors which contain both a cytoplasmic domain such as a CD3 chain of the TCR, for example zeta, as well as a cytoplasmic domain derived from a co-stimulatory molecule such as CD28, in a single chain to provide both a TCR activation signal and a co-stimulatory signal in the host cell.

The DNA encoding the co-stimulatory chimeric receptors associated with regulatory sequences that permit the transcription and translation of the receptor gene and its expression in a host cell is transduced into a host cell for production of the co-stimulatory chimeric receptor protein. The present invention further includes methods of using the co-stimulatory chimeric receptors for cell proliferation and as therapeutics for treating cancer and disease.

DESCRIPTION OF THE DRAWINGS

FIGS. 1A through 1D illustrate the structures of the co-stimulatory chimeric receptors of the invention.

FIG. 2 is a listing of oligonucleotides (SEQ ID NOS: 1–16) as described in the Examples, infra.

DETAILED DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The present invention discloses novel co-stimulatory chimeric receptor proteins and DNA sequences encoding these novel proteins.

These receptors may be introduced into host cells such as lymphocytes to augment proliferation of the cells in vitro and invivo. The receptors may be introduced into host cells already expressing a chimeric effector function receptor such as that described in U.S. Pat. No. 5,359,046, or the two types of receptors may be introduced together and co-expressed in the same host cell. Further aspects of the invention are discussed in detail below.

The delivery of co-stimulatory signals required for induction of effector function in T cells can be initiated by the co-stimulatory chimeric receptors of the invention having the ability to bind to different ligands. These co-stimulatory chimeric receptors can overcome the lack of counter-receptor expression by certain virally-infected cells, tumor cells, or otherwise potentially immunogenic target cells, and may avoid the possibility of inactivation of T cells upon encounter with soluble antigen (e.g., cell-free virus particles) in the absence of APCs.

Definitions

The term "effector function" refers to the specialized function of a differentiated cell. Effector function of a T cell, for example, may be cytolytic activity or helper activity including the secretion of cytokines.

The term "chimeric effector function receptor" refers to a chimeric receptor that comprises an extracellular domain, a transmembrane domain and a cytoplasmic domain as described in U.S. Pat. No. 5,359,046. The extracellular domain binds a ligand and transmits a signal to the cytoplasmic domain which transduces an effector function signal to the cell in which the receptor is expressed.

The term "effector function signal" refers to the effector function signal provided by the native T cell receptor (TCR) in a cell or by a chimeric effector function receptor protein expressed in a host cell upon binding of a ligand.

The term "co-stimulatory signal" refers to the activation signal generated by contact between a ligand and a co-stimulatory receptor molecule on a cell which acts in synergy with the primary effector function signal.

The term "co-stimulatory chimeric receptor" refers to a chimeric receptor that comprises an extracellular ligand binding domain, a transmembrane domain and a cytoplasmic co-stimulatory signaling domain. The extracellular domain and the cytoplasmic domain are not naturally found together on a single receptor protein.

In its general embodiments the present invention describes novel co-stimulatory chimeric receptor proteins, nucleic acid sequences encoding the receptors, the vectors containing the nucleic acid sequences encoding the receptors, host cells expressing the receptors and methods of using the receptors to augment effector function of cells, to augment lymphocyte proliferation, to protect lymphocytes from anergy. In one embodiment of the invention, a novel co-stimulatory chimeric receptor is provided containing an extracellular ligand binding domain and a cytoplasmic co-stimulatory signaling domain that do not naturally exist together as a single receptor protein.

The co-stimulatory chimeric receptors are designed for expression in cells, particularly lymphocytes, to augment proliferation and/or effector function of the cells in response to binding of a ligand to the extracellular domain of the chimeric receptor. The host cells bearing the receptors of the invention will expand in number (proliferate) in response to the binding of a ligand to the extracellular ligand binding domain. The extracellular domains include, but are not limited to cell surface differentiation antigens, e.g. CD4, CD8, etc., a secreted targeting molecule, e.g. interleukin-14 (IL-14), etc., a cell surface/secreted targeting molecule, e.g. an antibody (Ab) or single-chain antibody (Sab), antibody fragments, etc., a cell adhesion molecule, e.g. ICAM, LFA-1, etc., or portions or derivatives thereof.

The co-stimulatory chimeric receptor of the invention can be introduced and expressed in host cells containing native

T cell receptors or expressing chimeric effector function signaling receptors as described in U.S. Pat. No. 5,359,046 to augment effector function of the cells, for example to increase cytolytic activity, to increase cytokine production, to augment proliferation or to protect the cells from anergy. In addition, differentiation and/or maturation of the host cells, e.g. native T cells, may be augmented by the co-stimulatory chimeric receptors of the invention.

Alternatively, hybrid chimeric receptor molecules comprising an extracellular ligand binding domain, a transmembrane domain and a combined cytoplasmic domain comprising an effector function signaling domain, e.g. zeta, linked to a co-stimulatory signaling domain, e.g. CD28, are introduced into host cells (FIG. 1B). Upon introduction of these novel hybrid co-stimulatory/effector function chimeric receptors into cells, both a primary effector function signal and a co-stimulatory signal can be regulated by addition of a single ligand that binds to the extracellular domain of the hybrid receptor.

If the co-stimulatory chimeric receptor of the invention is expressed in host cells already expressing the chimeric effector function receptors of U.S. Pat. No. 5,359,046, for example the CD4/zeta chimeric receptor, then the effector function of the dual chimeric receptor expressing cells, e.g. cytotoxicity or cytokine production, can be activated and/or augmented upon addition of the same ligand. Alternatively, the ligand that binds to the extracellular binding domain of the chimeric effector function receptor may differ from the ligand that binds to the extracellular domain of the co-stimulatory chimeric receptor. In that case, an effector function signal will only result if both ligands bind to their respective extracellular domains. This can optimize specificity of response of the host cell because it will require two different target molecules to signal effector function. For example, where one target ligand is associated with a particular cancer cell and the other target ligand is associated with a particular cell type, then the host cell will be directed to function only when both cells types are present.

The cytoplasmic domain of the co-stimulatory chimeric receptor proteins may be derived from a protein which is known to activate various messenger systems. The protein from which the cytoplasmic domain is derived need not have ligand binding capability by itself, it being sufficient that such protein may associate with another protein providing such capability.

Cytoplasmic regions of interest include CD28 and CTLA-4, CD2, CD5, ICAM-1, Leukocyte Functional Antigen (LFA-1) (CD11a/CD18) and Heat Soluble Antigen (HSA), and such other cytoplasmic regions capable of transmitting a co-stimulatory signal as a result of interacting with other proteins that bind to a ligand. A number of cytoplasmic regions or functional fragments or mutants thereof may be employed, generally ranging from about 50 to 500 amino acids, where the entire naturally occurring cytoplasmic region may be employed or only an active portion thereof.

While usually the entire cytoplasmic region will be employed, in many cases, it will not be necessary to use the entire chain. To the extent that a truncated portion may find use, such truncated portions may be used in place of the intact chain.

Additionally, the chimeric receptors of the invention include hybrid receptors that contain a cytoplasmic domain of the co-stimulatory molecule and the entire or a portion of the cytoplasmic domain of the CD3 chains of the T cell receptor, for example the zeta, eta, delta, gamma or epsilon chains of the T cell receptor, or the beta and gamma chains of the FcεR1 receptor, B29, or a tyrosine kinase such as a member of the Syk tyrosine kinase family which activates cytolysis, Syk or ZAP-70, where the cytoplasmic domain is capable of activating effector function in a host cell. As an example, the C-terminus of a CD4/CD28 receptor is joined to the N-terminal residue of the cytoplasmic domain of zeta (amino acid residue 203 of CD28 cytoplasmic domain joined to residue 31 of the zeta cytoplasmic domain) by oligonucleotide directed splicing, resulting in a chimeric molecule with the extracellular and transmembrane portions of CD4 and the cytoplasmic domains of CD3-zeta and CD28 linked head-to-tail (see FIG. 1). Thus, binding of the appropriate ligand, e.g. gp120 to the extracellular domain (CD4) results in the transduction of both a primary activation signal and a co-stimulatory signal simultaneously.

The transmembrane domain may be contributed by the domain of the protein contributing the cytoplasmic portion, the domain of the protein contributing the extracellular portion, or a domain associated with a totally different protein. In some cases, it will be convenient to have the transmembrane domain naturally associated with one or the other of the other domains, particularly the extracellular domain. In some cases it will be desirable to employ the transmembrane domain of the zeta, eta or FcεR1γ chains or related proteins or of the co-stimulatory proteins, for example CD28 or CTLA-4, which contain a cysteine residue capable of disulphide bonding, so that the resulting chimeric protein will be able to form disulphide linked dimers with itself, or with unmodified versions of the chains or co-stimulatory proteins. In some instances, the transmembrane domain will be selected to avoid binding of such domain to the transmembrane domain of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex. In other cases it will be desirable to employ the transmembrane domain of zeta, eta, FcεR1γ1 or the co-stimulatory protein, in order to retain physical association with other cell surface receptors or proteins.

The extracellular domain may be obtained from any of the wide variety of extracellular domains or secreted proteins associated with ligand binding and/or signal transduction. The extracellular domain may be part of a protein which is monomeric, homodimeric, heterodimeric, or associated with a larger number of proteins in a non-covalent or disulfide-bonded complex.

In particular, the extracellular domain may consist of monomeric or dimeric immunoglobulin (Ig) molecules, or portions or modifications thereof, which are prepared in the following manner. The full-length IgG heavy chain comprising the VH, CH1, hinge, and the CH2 and CH3 (Fc) Ig domains is fused to the co-stimulatory cytoplasmic signalling domain via the appropriate transmembrane domain. If the VH domain alone is sufficient to confer antigen-specificity (so-called "single-domain antibodies"), homodimer formation of the Ig-co-stimulatory chimera is expected to be functionally bivalent with regard to antigen binding sites. If both the VH domain and the VL domain are necessary to generate a fully active binding site, both the IgH-co-stimulatory molecule and the full-length IgL chain are introduced into cells to generate an active antigen-binding site. Dimer formation resulting from the intermolecular Fc/hinge disulfide bonds results in the assembly of Ig-co-stimulatory chimeric receptors with extracellular domains resembling those of IgG antibodies. Derivatives of these chimeric receptors include those in which only portions of the heavy chain are employed in the fusion. For example, the VH domain (and the CH1 domain) of the heavy chain can be retained in the extracellular domain of the

Ig-co-stimulatory chimera, but VH-dimers are not formed. As above, the full-length IgL chain can be introduced into cells to generate an active antigen-binding site.

The extracellular domain may consist of an Ig heavy chain which in turn may be covalently associated with an Ig light chain by virtue of the presence of the CH1 region, or may become covalently associated with other Ig heavy/light chain complexes by virtue of the presence of hinge, CH2 and CH3 domains. The two heavy/light chain complexes may have different specificities, thus creating a chimeric receptor which binds two distinct antigens. Depending on the function of the antibody, the desired structure and the signal transduction, the entire chain may be used or a truncated chain may be used, where all or a part of the CH1, CH2 or CH3 domains may be removed or all or part of the hinge region may be removed.

Because association of both the heavy and light V domains are required to generate a functional antigen binding site of high affinity, in order to generate an Ig chimeric receptor with the potential to bind antigen, a total of two molecules will typically need to be introduced into the host cell. Therefore, an alternative and preferred strategy is to introduce a single molecule bearing a functional antigen binding site. This avoids the technical difficulties that may attend the introduction and coordinated expression of more than one gene construct into host cells. This "single-chain antibody" (SAb) is created by fusing together the variable domains of the heavy and light chains using an oligo- or polypeptide linker, thereby reconstituting an antigen binding site on a single molecule.

Single-chain antibody variable fragments (SAbFv) in which the C-terminus of one variable domain (VH or VL) is tethered to the N-terminus of the other (VL or VH, respectively), via a oligo- or polypeptide linker, have been developed without significantly disrupting antigen binding or specificity of the binding (Bedzyk et al., *J. Biol. Chem.*, 265:18615 (1990); Chaudhary et al., *Proc. Natl. Acad. Sci.* 87:9491 (1990)). The SAbFvs used in the present invention may be of two types depending on the relative order of the VH and VL domains: VH-l-VL or VL-l-VH (where "l" represents the linker). These SAbFvs lack the constant regions (Fc) present in the heavy and light chains of the native antibody. In another aspect of the present invention, the SAbFv fragment may be fused to all or a portion of the constant domains of the heavy chain, and the resulting extracellular domain is joined to the cytoplasmic domain via an appropriate transmembrane domain that will permit expression in the host cell. The resulting chimeric receptors differ from the SAbFvs, described above, in that upon binding of antigen they initiate signal transduction via their cytoplasmic domain.

To aid in the proper folding and efficient expression of the chimeric receptors, the antibody-derived extracellular domains may be connected at their C-terminal end to one of a number of membrane hinge regions which are a normal part of membrane-bound immunoglobulin molecules. For example, the eighteen amino acids of the IGHG3 M1 exon may be used (Bensmana and Lefranc, *Immunogenet.* 32:321–330 (1990)). The TM domain is attached to the C-terminal end of the membrane hinge. It is also contemplated that membrane hinge sequences may be used to connect non-antibody derived extracellular domains to the transmembrane domains to increase chimeric receptor expression.

Various naturally occurring receptors may also be employed as extracellular domains, where the receptors are associated with surface membrane proteins, including cell differentiation (CD) antigens such as CD4, CD8, cytokine or hormone receptors or adhesion molecules. The receptor may be responsive to a natural ligand, an antibody or fragment thereof, a synthetic molecule, e.g., drug, or any other agent which is capable of inducing a signal. Thus, in addition to CD receptors, ligands for receptors expressed on cancer cells could supply the extracellular domain of the chimeric receptors of the invention. For example human Heregulin (Hrg) a protein similar in structure to Epidermal Growth Factor (EGF), has been identified as a ligand for the receptor Her_2 which is expressed on the surface of breast carcinoma cells and ovarian carcinoma calls (Holmes et al., *Science* 256:1205–1210 (1992)). The murine equivalent is the "Neu" protein (Bargman et al., *Nature* 319:226–230 (1986)). The extracellular domain of Hrg could be joined to the CD28 or CD4 transmembrane domain and the CD28 co-stimulatory receptor cytoplasmic domain to form a chimeric construct of the invention to augment the effector function of T cells to kill breast carcinoma cells. In addition, either member of a ligand/receptor pair, where one is expressed on a target cell such as cancer cell, a virally infected cell or an autoimmune disease cause cell may be used as an extracellular domain in the present invention. Moreover, receptor-binding domains of soluble protein ligands or portions thereof could be used as extracellular domains. Binding portions of antibodies, cytokines, hormones or serum proteins can also be used. Alternatively, soluble components of the cytokine receptors such as IL-6R, IL-4R and IL-7R can form the extracellular domains (Boulay and Paul, *Current Biology* 3:573–581 (1993)).

In addition, "hybrid" extracellular domains can be used. For example, two or more antigen-binding domains from antibodies of different specificities, two or more different ligand-binding domains, or a combination of these domains can be connected to each other by oligo- or polypeptide linkers to create multispecific extracellular ligand binding domains. These domains can be used to create co-stimulatory chimeric receptors of the invention which will respond to two or more different extracellular ligands. The extracellular domain may consist of a CD receptor, such as CD4, joined to a portion of an immunoglobulin molecule, for example the heavy chain of Ig.

Where a receptor is a molecular complex of proteins, where only one chain has the major role of binding to the ligand, it will usually be desirable to use solely the extracellular portion of the ligand binding protein. Where the extracellular portion may complex with other extracellular portions of other proteins or form covalent bonding through disulfide linkages, one may also provide for the formation of such dimeric extracellular region. Also, where the entire extracellular region is not required, truncated portions thereof may be employed, where such truncated portion is functional. In particular, when the extracellular region of CD4 is employed, one may use only those sequences required for binding of gp120, the HIV envelope glycoprotein. In the case in which Ig is used as the extracellular region, one may simply use the antigen binding regions of the antibody molecule and dispense with the constant regions of the molecule (for example, the Fc region consisting of the CH2 and CH3 domains).

In some instances, a few amino acids at the joining region of the natural protein may be deleted, usually not more than 30, more usually not more than 20. Also, one may wish to introduce a small number of amino acids at the borders, usually not more than 30, more usually not more than 20. The deletion or insertion of amino acids will usually be as a result of the needs of the construction, providing for

convenient restriction sites, ease of manipulation, improvement in levels of expression, or the like. In addition, one may wish to substitute one or more amino acids with a different amino acid for similar reasons, usually not substituting more than about five amino acids in any one domain. The cytoplasmic domain will generally be from about 50 to 500 amino acids, depending upon the particular domain employed. The transmembrane domain will generally have from about 25 to 50 amino acids, while the extracellular domain will generally have from about 50 to 500 amino acids.

Normally, the signal sequence at the 5' terminus of the open reading frame (ORF) which directs the chimeric protein to the surface membrane will be the signal sequence of the extracellular domain. However, in some instances, one may wish to exchange this sequence for a different signal sequence. However, since the signal sequence will be removed from the protein, being processed while being directed to the surface membrane, the particular signal sequence will normally not be critical to the subject invention. Similarly, associated with the signal sequence will be a naturally occurring cleavage site, which will also normally be the naturally occurring cleavage site associated with the signal sequence or the extracellular domain.

Ligands for binding to the extracellular domain can be antigens including viral proteins, e.g. gp120 and gp41 envelope proteins of HIV, envelope proteins from the Hepatitis B and C viruses, the gB and other envelope glycoproteins of human cytomegalovirus (CMV), the envelope proteins from the Kaposi's sarcoma-associated herpes virus, and surface proteins found on cancer cells, e.g. the IL-14 receptor, CD19 and CD20 for B cell lymphoma, the Lewis Y and CEA antigens for a variety of carcinomas, the Tag72 antigen for breast and colorectal cancer, EGF-R for lung cancer and the HER-2 proteins which is often amplified in human breast and ovarian carcinomas. For other receptors, the receptors and ligands of particular interest are CD4, where the ligand is the HIV gp120 envelope glycoprotein, and other viral receptors, for example ICAM, which is the receptor for the human rhinovirus, and the related receptor molecule for poliovirus.

The chimeric constructs, which encode the chimeric receptor protein according to this invention are prepared in conventional ways. Since, for the most part, natural sequences may be employed, the natural genes may be isolated and manipulated, as appropriate, so as to allow for the proper joining of the various domains. Thus, one may prepare the truncated portion of the sequence by employing the polymerase chain reaction (PCR), using appropriate primers which result in deletion of the undesired portions of the gene. Alternatively, one may use primer repair, where the sequence of interest may be cloned in an appropriate host. In either case, primers may be employed which result in termini, which allow for annealing of the sequences to result in the desired open reading frame encoding the chimeric protein. Thus, the sequences may be selected to provide for restriction sites which are blunt-ended, or have complementary overlaps. During ligation, it is desirable that hybridization and ligation does not recreate either of the original restriction sites.

If desired, the extracellular domain may also include the transcriptional initiation region, which will allow for expression in the target host. Alternatively, one may wish to provide for a different transcriptional initiation region, which may allow for constitutive or inducible expression, depending upon the target host, the purpose for the introduction of the subject chimeric protein into such host, the level of expression desired, the nature of the target host, and the like. Thus, one may provide for expression upon differentiation or maturation of the target host, activation of the target host, or the like.

A wide variety of promoters have been described in the literature, which are constitutive or inducible, where induction may be associated with a specific cell type or a specific level of maturation. Alternatively, a number of viral promoters are known which may also find use. Promoters of interest include the β-actin promoter, SV40 early and late promoters, immunoglobulin promoter, human cytomegalovirus promoter, and the Friend spleen focus-forming virus promoter. The promoters may or may not be associated with enhancers, where the enhancers may be naturally associated with the particular promoter or associated with a different promoter.

The sequence of the open reading frame may be obtained from genomic DNA, cDNA, or be synthesized, or combinations thereof. Depending upon the size of the genomic DNA and the number of introns, one may wish to use cDNA or a combination thereof. In many instances, it is found that introns stabilize the mRNA. Also, one may provide for non-coding regions which stabilize the mRNA.

A termination region will be provided 3' to the cytoplasmic domain, where the termination region may be naturally associated with the cytoplasmic domain or may be derived from a different source. For the most part, the termination regions are not critical and a wide variety of termination regions may be employed without adversely affecting expression.

The various manipulations may be carried out in vitro or may be introduced into vectors for cloning in an appropriate host, e.g., *E. coli*. Thus, after each manipulation, the resulting construct from joining of the DNA sequences may be cloned, the vector isolated, and the sequence screened to insure that the sequence encodes the desired chimeric protein. The sequence may be screened by restriction analysis, sequencing, or the like. Prior to cloning, the sequence may be amplified using PCR and appropriate primers, so as to provide for an ample supply of the desired open reading frame, while reducing the amount of contaminating DNA fragments which may have substantial homology to the portions of the entire open reading frame.

The chimeric construct may be introduced into the host cell in any convenient manner. Techniques include calcium phosphate or DEAE-dextran mediated DNA transfection, electroporation, protoplast fusion, liposome fusion, biolistics using DNA-coated particles, transfection, and infection, where the chimeric construct is introduced into an appropriate virus, e.g. retrovirus, adenovirus, adeno-associated virus, Herpes virus, Sindbis virus, papilloma virus, particularly a non-replicative form of the virus, or the like. In addition, direct injection of naked DNA or protein- or lipid-complexed DNA may also be used to introduce DNA into cells.

Once the target host cell has been transformed, integration will usually result. However, by appropriate choice of vectors, one may provide for episomal maintenance. A large number of vectors are known which are based on viruses, where the copy number of the virus maintained in the cell is low enough to maintain the viability of the cell. Illustrative vectors include SV40, EBV and BPV.

Once one has established that the transformed host cell expresses the chimeric protein as a surface membrane protein in accordance with the desired regulation and at a desired level, one may then determine whether the transmembrane protein is functional in the host to provide for the desired co-stimulatory signal induction.

The effects of co-stimulation in lymphocytes can be measured by a variety of techniques known to those skilled in the art. For example, augmentation of proliferation can be determined by measuring the incorporation of either tritiated thymidine or orotic acid to measure DNA synthesis following ligand binding to the co-stimulatory chimeric receptor of the invention. The incorporation of bromodeoxyuridine into newly synthesized DNA can be measured by immunological staining and the detection of dyes, or by ELISA (Enzyme-linked immunosorbent assay) (Doyle et al., *Cell and Tissue Culture: Laboratory Procedures*, Wiley, Chichester, England, (1994)). The mitotic index of cells can be determined by staining and microscopy, by the fraction labeled mitoses method or by FACS analysis (Doyle et al., supra, (1994); Dean, *Cell Tissue Kinet.* 13:299–308 (1980); Dean, *Cell Tissue Kinet.* 13:672–681 (1980)). The increase in cell size which accompanies progress through the cell cycle can be measure by centrifugal elutriation (Faha et al., *J. Virol.* 67:2456–2465 (1993)). Increases in the number of cells may also be measured by counting the cells, with or without the addition of vital dyes. In addition, signal transduction can also be measured by the detection of phosphotyrosine, the in vitro activity of tyrosine kinases from activated cells, c-myc induction, and calcium mobilization.

TCR binding results in the induction of CD69 expression and cytokine secretion. The ability of a co-stimulatory chimeric receptor of the invention to provide co-stimulation with suboptimal doses of a ligand that stimulates the TCR, is measured by a restoration of CD69 induction and augmented cytokine secretion as described, for example, in the examples, infra.

The subject chimeric receptors may be used to augment the proliferation, and effector function (including cytolysis and cytokine secretion) of immune cells and increase resistance of those cells to anergy. For example, the co-stimulatory chimeric receptors of the invention can be used to deliver cytokines in vitro and in vivo. One measure of T cell activation is the production of cytokines. This is true for both CD4 and CD8 T cells. Moreover, cytokine production is also susceptible to anergy when T cells are stimulated via the TCR without co-stimulation through CD28. Another aspect of CD28 co-stimulation is its ability to augment cytokine production by increasing transcription of cytokine genes and stabilizing cytokine mRNAs. CD4+ T cells in addition to CD8+ T cells expressing chimeric receptors may be of particular interest because they have a greater capacity for cytokine production. Any number of cytokines are of interest because of their potential relationships to certain disease states, but in particular the presence of IL-2, IL-4, and γ-IFN production is important because of their immunomodulatory activities, TNF for its anti-tumor effects, and IFN-α for its anti-viral effects.

The chimeric receptors may be introduced into cells that already contain a chimeric effector function receptor that stimulates effector function upon contact with a target ligand. The two chimeric constructs may respond to the same or different ligands. Alternatively, a hybrid co-stimulatory chimeric receptor may be used which contains both a co-stimulatory signaling domain and an effector function signaling domain. These cells would respond to a single target ligand by proliferating, expressing effector functions such as cytolysis and cytokine production and demonstrating increased resistance to anergy. Thus, these lymphocytes can be activated by any group of cells which contain specific membrane proteins or antigens which may be distinguished from the membrane proteins or antigens on normal cells. For example, neoplastic cells, virus-infected cells, parasite-infected cells, or any other diseased cells would be targets for receptor-containing lymphocytes.

Among the lymphocytes which can be used to treat human disease are cytotoxic CD8+ T cells (CTLs) which have been engineered with chimeric receptors containing extracellular domains which recognize specific antigens and can be used to augment proliferation and/or killing of infected cells in a variety of viral, and parasitic diseases, where the infected cells express the antigens from the pathogen. In particular, co-stimulatory chimeric receptor-CTLs would be particularly effective against viral diseases where transplanted autologous CTLs have shown some efficacy, such as CMV (Reusser et al., *Blood* 78:1373–1380 (1991), Riddell et al., *Science* 257:238–241 (1992)) or where explanted and expanded CTLs continued to have cytolytic activity against virally infected cells, such as HIV (Lieberman et al., *Aids Res. and Human Retroviruses* 11:257–271(1995)). These chimeric receptors can be constructed with extracellular domains which recognize the viral envelope proteins. For example, SAbs which recognize either gp120 or gp41, or the CD4 extracellular domain which recognizes gp120 can be used to engineer HIV-specific CTLs. Chimeric receptor-CTLs can also be engineered for use against other viruses, such as Hepatitis B virus, Hepatitis C virus, Kaposi's sarcoma associated Herpes virus, the Herpes Simplex viruses, Herpes Zoster virus, and papilloma viruses. Another target for the engineered CTLs are neoplastic cells which express cancer-specific neoantigens or over-express specific membrane proteins. Examples include the IL-14 receptor, CD19 and CD20 for B cell lymphoma, the Lewis Y and CEA antigens for a variety of carcinomas, the Tag72 antigen for breast and colorectal cancer, EGF-R for lung cancer, and the HER-2 protein which is often amplified in human breast and ovarian carcinomas. Chimeric receptor-CTLs can also be used to target autoimmune cells in the treatment of autoimmune diseases such as Systemic Lupus Erythematosis (SLE), *myasthenia gravis*, diabetes, rheumatoid arthritis, and Grave's disease.

$CD4^+$ helper T cells (THs) engineered with chimeric receptors containing extracellular domains which recognize specific antigens can also be used to treat human disease. In particular, lymphokine production by chimeric receptor-THs may be effective against cancer cells and mycobacterial infections, including *Mycobacterium avium*, *Mycobacterium tuberculosis* and *Mycobactium leprae*.

Various cell types containing the chimeric constructs described above may be grown in an appropriate nutrient medium for expansion or may be expanded directly in the body via signaling through the chimeric receptors, depending on the cell type, and used in a variety of ways.

Additional types of cells that would benefit from the introduction of the chimeric receptors of the invention include cells that have genes previously introduced or simultaneously introduced with a chimeric receptor which may serve in protein production or to correct a genetic defect. Production of proteins may include growth factors, such as, erythropoietin, G-CSF, M-CSF, and GM-CSF, epidermal growth factor, platelet derived growth factor, human growth factor, transforming growth factor, etc; lymphokines, such as the interleukins.

The recipient of genetically modified allogeneic cells can be immunosuppressed to prevent the rejection of the transplanted cells. In the case of immunocompromised patients, no pretransplant therapy may be required. Another alternative source of cells to be transplanted are so-called "universal donor" cells which have been genetically engineered so

that they do not express antigens of the major histocompatibility complex or molecules which function in antigen presentation.

High-titer retroviral producer lines are used to transduce the co-stimulatory chimeric receptor constructs into autologous or allogeneic human T-cells, hematopoietic stem cells or other cells, described above through the process of retroviral mediated gene transfer as described by Lusky et al. in *Blood* 80:396 (1992).

A wide variety of target hosts may be employed, normally cells from vertebrates, more particularly, mammals, desirably domestic animals or primates, particularly humans. The subject chimeric constructs may be used for the investigation of particular pathways controlled by signal transduction, for initiating cellular responses employing different ligands, for example, for inducing activation of a particular subset of lymphocytes, where the lymphocytes may be activated by particular surface markers of cells, such as neoplastic cells, virally infected cells, or other diseased cells, which provide for specific surface membrane proteins which may be distinguished from the surface membrane proteins on normal cells. The cells may be further modified so that expression cassettes may be introduced, where activation of the genetically modified cell will result in secretion of a particular product. In this manner, one may provide for directed delivery of specific agents, such as interferons, TNF's, perforans, naturally occurring cytotoxic agents, or the like, where the level of secretion can be greatly enhanced over the natural occurring secretion. Furthermore, the cells may be specifically directed to the site using injection, catheters, or the like, so as to provide for localization of the response.

The subject invention may find application with effector cells such as lymphocytes including cytotoxic lymphocytes (CTL), Natural killer cells (NK), tumor-infiltrating-lymphocytes (TIL) or other cells which are capable of releasing cytokines or killing target cells when activated. Thus, diseased cells, such as cells infected with HIV, HTLV-I or II, cytomegalovirus, hepatitis B or C virus, mycobacterium avium, etc., or neoplastic cells, where the diseased cells have a surface marker associated with the diseased state may be made specific targets of the effector cells. In particular, diseased cells that lack the appropriate co-stimulatory ligands are targets for the cells expressing the co-stimulatory chimeric receptors of the invention. By providing a receptor extracellular domain, e.g., CD4, which binds to a surface marker of the pathogen or neoplastic condition, e.g., gp120 for HIV, the cells may serve as therapeutic agents. By modifying the cells further to prevent the expression or translocation of functional Class I and/or II MHC antigens, the cells will be able to avoid recognition by the host immune system as foreign and can therefore be therapeutically employed in any individual regardless of genetic background. Alternatively, one may isolate and transfect host cells with the subject constructs and then return the transfected host cells to the host.

Other applications include transduction of host cells from a given individual with retroviral vector constructs directing the synthesis of the chimeric construct. By transduction of such cells and reintroduction into the patient one may achieve autologous gene therapy applications.

In addition, suitable host cells include hematopoietic stem cells, which develop into effector cells with both myeloid and lymphoid phenotype including granulocytes, mast cells, basophils, macrophages, natural killer (NK) cells and T and B lymphocytes. Introduction of the chimeric constructs of the invention into hematopoietic stem cells thus permits the induction of effector functions such as cytotoxicity, cytokine production, proliferation and differentiation of various cell types derived from hematopoietic stem cells providing a continued source of effector cells to fight various diseases. The zeta subunit of the T cell receptor is associated not only with T cells, but is present in other cytotoxic cells derived from hematopoietic stem cells. Therefore, because stem cells transplanted into a subject via a method such as bone marrow transplantation exist for a lifetime, a continued source of effector cells is produced by introduction of the chimeric receptors of the invention into hematopoietic stem cells. Effector cells derived from gene-modified stem cells will express a chimeric effector function and/or a co-stimulatory chimeric receptor, and will be expected to have enhanced capacity to fight virally infected cells, cells expressing tumor antigens, cells responsible for autoimmune disorders, and have increased resistance to anergy. Additionally, introduction of the chimeric receptors into stem cells with subsequent expression by both myeloid and lymphoid cytotoxic cells may have certain advantages in immunocompromised individuals such as patients with AIDS. This is because the maintenance of the lymphoid cytotoxic cells ($CD8^+$) and the continued function of helper T cells ($CD4^+$) may be impaired in AIDS patients due to a failure of natural co-stimulatory mechanisms.

The chimeric receptor constructs of the invention are introduced into hematopoietic stem cells followed by bone marrow transplantation to permit expression of the chimeric receptors in all lineages derived from the hematopoietic system. High-titer retroviral producer lines are used to transduce the chimeric receptor constructs, for example CD4/CD28, into both murine and human T-cells and human hematopoietic stem cells through the process of retroviral mediated gene transfer as described by Lusky et al. in *Blood* 80:396 (1992). For transduction of hematopoietic stem cells, the bone marrow is harvested using standard medical procedures and then processed by enriching for hematopoietic stem cells expressing the CD34 antigen as described by Andrews et al. in *J. Exp. Med.* 169:1721 (1989). These cells are then incubated with the retroviral supernatants in the presence of hematopoietic growth factors such as stem cell factor and IL-6. The bone marrow transplant can be autologous or allogeneic, and depending on the disease to be treated, different types of conditioning regimens are used (see, Surgical Clinics of North America 66:589 (1986)). The recipient of the genetically modified stem cells can be treated with total body irradiation, chemotherapy using cyclophosphamide, or both to prevent the rejection of the transplanted bone marrow. In the case of immunocompromised patients, no pretransplant therapy may be required because there is no malignant cell population to eradicate and the patients cannot reject the infused marrow. In addition to the gene encoding the chimeric receptor, additional genes may be included in the retroviral construct.

The following examples are by way of illustration and not by way of limitation.

EXPERIMENTAL

EXAMPLE 1

Construction of CD4–CD28 Chimeric Receptor

PCR was used to amplify the extracellular and transmembrane portions of human CD28 from a cDNA library of the human T cell line Jurkat (ClonTech) using oligonucleotides 3 and 4 (SEQ ID NO:3 and SEQ ID NO:4) (FIG. 2). A DNA fragment containing the human CD4 gene was obtained by

digesting the plasmid pIK1.1CD4 a plasmid described in U.S. Pat. No. 5,359,046 to Capon et al. with EcoRI and NarI. The CD28 fragment was digested with BamHI and NarI, and the two fragments were ligated together and inserted into the pIK1.1 plasmid as described in U.S. Pat. No. 5,359,046, which had been cut with EcoRI and Bgl II by simultaneous double ligation. This yielded a new plasmid, pIK1.1CD4–CD28 which contained the entire coding sequence of human CD4 juxtaposed to the coding sequence of human CD28. The integrity of the new construct was verified by DNA sequencing (Sanger et al., *Proc. Natl. Acad. Sci. USA* 74:5463–5467 (1977)). From this template the CD4/CD28 chimeric receptors were made by oligonucleotide directed mutagenesis (Zoller and Smith, *Nucleic Acids Res.* 10:6487–6500) (1982). Oligonucleotides were designed to create new junctions at the desired sites within the CD4 and CD28 cDNAs (FIG. 2). Using this technique three CD4/CD28 chimeric sequences were created: CH28-1 consists of the extracellular and transmembrane domains of human CD4 fused to the cytoplasmic domain of human CD28 (oligonucleotides 10 and 11 (SEQ ID NO:10 and SEQ ID NO:11)); CH28-2 consists of the extracellular domain of CD4 fused to the transmembrane and cytoplasmic domains of CD28 (oligonucleotide 5 (SEQ ID NO:5)); and CH28-3 consists of the extracellular domain of CD4 fused to a portion of the extracellular domain plus the entire transmembrane and cytoplasmic domains of CD28 (oligonucleotide 6 SEQ ID NO:6)). These constructs are depicted in FIG. 1A. The portion of the extracellular domain of CD28 contains a cysteine residue (position 141) which is important for the formation of homodimers of CD28 at the cell surface. The pIK1.1 plasmid vectors containing the CD4/CD28 fusions (CH28-1, 2, 3) and a selectable marker were created by blunt-end cloning of a HincII fragment of pUCRNeoG (Hudziak et al., *Cell* 31:137–146 (1982)) containing the neomycin phosphotransferase (neo^r) gene into pIKCH28-1, pIKCH28-2, and pIKCH28-3 which were cut with SspI. The resulting vectors contain the CD4/CD28 chimeric sequences whose expression is driven by a CMV promoter and the neo^r gene driven by the RSV LTR. This vector will confer resistence to the drug GD18 when expressed in eukaryotic cells.

Construction of CD8-zeta chimeric receptors

Plasmid vectors encoding CD8-zeta chimeric receptors were created by oligonucleotide-directed mutagenesis using as templates DNA encoding the human CD8α gene (ATCC No. 59565, *Mol. Cell. Biol.* 8:2837–2947 (1988)) and the plasmid CD4-F3 (pIK1.1.CD4-F3) chimeric receptor as described in U.S. Pat. No. 5,359,046. A fragment containing the CD8α cDNA was amplified from the plasmid EBO-pCD.Leu2 using oligonucleotides 12 and 13 (SEQ ID NO:12 and SEQ ID NO:13) (FIG. 2). The PCR product was cut with BstEII and EcoRI to give a 2.3 kb fragment containing the entire coding sequence for CD8α. This fragment was inserted between the BstEII and EcoRI sites of pIK1.1CD4-F3 to generate the plasmid pIK1.1CD8-CD4-F3. Oligonucleotides were designed to create a new junction between CD8α and CD4 sequences at the start of the CD4 transmembrane region and the human CD8α C-terminus of the extracellular region, giving rise to the CD8-F3/A construct (oligonucleotide 14 (SEQ ID NO:14), FIG. 2). In like manner, CD8-F3/B (oligonucleotide 15 (SEQ ID NO:15)) was created by joining the CD8α transmembrane domain directly to the zeta cytoplasmic domain. Thus, the CD8-F3/A chimeric receptor contains the CD8α extracellular domain, the transmembrane domain of CD4, and the cytoplasmic domain of CD3 zeta. CD8-F3/B consists of the extracellular and transmembrane domains of CD8α and the cytoplasmic domain of CD3 zeta. The CD8-F2 chimeric sequence was created in like manner by inserting an EcoRI-BstEII fragment containing CD8-F3 into the vector pIK1.1.CD4-F2 as described in U.S. Pat. No. 5,359,046 and using oligonucleotide-directed mutagenesis (oligonucleotide 16 (SEQ ID NO:16), FIG. 2) to create a chimeric sequence where the extracellular domain of CD8α is fused to the transmembrane and cytoplasmic domains of CD3 zeta. The CD8-F2, CD8-F3/A, and CD8-F3/B sequences were then cloned into the EBV-based expression vector p220.2pIK1.1F3 by replacing a HindIII-SfiI fragment of p220.2pIK1.1. F3 containing the F3 sequences with HindIII-SfiI fragments containing CD8-F2, CD8-F3/A, or CD8-F3/B. The p220.2pIK1.1. F3 plasmid was constructed by first inserting a HindIII-AvrII fragment of pIK1.1 between the HindIII and XbaI sites of p220.2 (Yates et al., *Nature* 313:812–815 (1985)) to give p220.2pIK1.1, and then inserting a HindIII-SfiI fragment from pIK1.1.F3 between the HindIII and SfiI sites of p220.2pIK1.1 to give p220.2pIK1.1.F3. The CD8-zeta constructs are depicted in FIG. 1B.

Antibodies

The OKT3, OKT4A, and OKT8 monoclonal antibodies to human CD3, CD4, and CD8α respectively, were obtained from Ortho Diagnostics Systems, Raritan, N.J. Leu3A monoclonal antibodies which recognize human CD4 and the Leu23 antibody to human CD69 were obtained from Becton-Dickinson Immunocytometry Systems, San Jose, Calif. The antibody W6/32 recognizes an invariant determinant expressed on human HLA Class I antigens. The mouse IgG2a myeloma protein (Litton Bionetics, Kensington, Md.) was used as a control antibody for FACS analysis.

Cell lines and Transfections

The human leukemic T cell line Jurkat was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), glutamine, penicillin, and streptomycin). Jurkat cell lines and clones transfected with the CH28 vectors (CH28-1, -2, -3 and CH2-1, CH2-2 and CH2-3) were passaged in the above medium with the addition of G418 (Geneticin, Gibco, Grand Island, N.Y.) at 2 mg/ml. Electroporation of the pIK1.1.CH28-neo and p220pIK1.1.CD8 (CD8-zeta) vectors into Jurkat T cells was performed in a Gene Pulser (Bio-Rad, Richmond, Calif.) using a voltage of 250 V and a capacitance of 960 μF with 20 μg of plasmid DNA per 10^7 cells. After transfection with pIKCH28-neo vectors, cells were grown for two days in RPMI before transferring to G418-containing medium. Clones of transfected cells were obtained by limiting dilution and screened for CD8 or CH28 (CD4) surface expression by flow cytometry (see below). Jurkat cells transfected with the CD8-zeta vector were passaged in 200 μg/ml hygromycin-containing medium and cloned by limiting dilution.

Flow Cytometry

Approximately $1\times10_6$ cells/condition were stained with saturating concentrations of antibodies directly conjugated to the fluorochrome fluorescein isothiocyanate or with unlabeled antibodies followed by a fluorescein-conjugated goat anti-mouse IgG, and analyzed using a Becton-Dickinson FACScan instrument.

CD4–CD28 chimeric receptors provide co-stimulation for CD3/TCR stimulation in Jurkat T cells

The chimeric CD4/CD28 constructs described above were transfected by electroporation into Jurkat cells and the transfected cells were selected in G418 and cloned by limiting dilution. Expression of the CD4/CD28 chimeric receptor on Jurkat cell clones was quantified by flow cytom-

etry using anti-CD4 antibodies. Jurkat T cell clones expressing comparable levels of each of the CD4/CD28 chimeric receptors, CH28-1, CH28-2, and CH28-3, were obtained. The ability of the CH28 chimeric receptors to function as co-stimulatory molecules was evaluated using the induction of the T cell activation antigen CD69 as an indicator (Testi et al., *J. Immunol.* 142:1854–1860). Flow cytometry revealed a very low degree of basal CD69 expression on non-stimulated cells. Maximal levels were induced on all cells with phorbol myristate acetate (PMA), an activator of protein kinase C. Stimulation of the TCR by anti-CD3 antibodies also resulted in the induction of CD69 expression on Jurkat T cells. At concentrations of anti-CD3 below 25 ng/ml no induction of CD69 was observed. Under these conditions addition of anti-CD4 antibodies, which stimulate the CD4/CD28 chimeric receptor, restored the induction of CD69 expression. Stimulation of the CD4/CD28 receptor alone did not induce CD69 expression, nor did the control antibody, W6/32. These data show that all three of the CD4/CD28 chimeric receptors (CH28-1, CH28-2, CH28-3) function as co-stimulatory molecules together with CD3/TCR stimulation in Jurkat T cells.

Function of the CD4/CD28 chimeric receptor in Jurkat cells expressing a zeta chimeric receptor

To test whether the CD4/CD28 chimeric receptor could provide co-stimulation for zeta chimeric receptor stimulation, Jurkat T cells expressing the CH28-1 chimeric receptor (Jurkat CH28-1 clone #2) were transfected with the CD8-zeta chimeric receptors described above. Transfected Jurkat CH28-1 cells were selected in hygromycin, cloned by limiting dilution, and analyzed for CD8-zeta expression by flow cytometry using anti-CD8 antibodies (OKT8). Jurkat CH28-1 cells expressing each of the three CD8-zeta receptors were obtained. One clone expressing CD8-F2 (F10) and one expressing CD8-F3/B (B6) were evaluated for co-stimulation by the chimeric CD4/CD28 receptor. The ability of the CH28-1 receptor to provide co-stimulation with suboptimal doses of anti-CD3 (TCR stimulation) and anti-CD8 antibodies (CD8-zeta stimulation) were compared. Anti-CD4 treatment restored CD69 induction in CH28-1/CD8-zeta expressing Jurkat cells stimulated with a suboptimal dose of anti-CD3. Moreover, anti-CD4 antibodies also restored CD69 induction in cells stimulated with low doses of anti-CD8. These results demonstrate that CD4/CD28 chimeric receptors can provide co-stimulation for zeta-based chimeric receptors as well as for CD3/TCR stimulation. Thus, chimeric receptors employing the cytoplasmic domain of CD28 can be used in combination with chimeric effector signal function receptors, e.g. CD4/zeta, to generate cells with two functional signaling receptors providing both of the signals necessary for optimal T cell activation.

EXAMPLE 2

Function of CD4/CD28 chimeric receptor in primary human CD8$^+$ T cell lines

CD4/CD28 chimeric receptors were introduced into primary human CD8+ T cells using retrovirus vectors. To accomplish this, the CH28-3 chimeric sequence was cloned into the retrovirus vector pRTD2.2svg. F3 (e$^-$) (Finer et al. (1994) Blood 83:43) to create the retroviral vector pRTD2.2svg. CH28-3 (e$^-$). This vector has the retroviral genomic RNA expression driven by a CMV promoter, and CH28-3 expression driven by the MMLV LTR. pRTD2.2svg. CH28-3 DNA. Primary human CD8+ T cells in active growth are transduced with the CH28-3 gene by the Kat retroviral transduction packaging system (Finer et al., *Blood*, supra).

Transduced T cells were analyzed for expression of the CD4/CD28 chimeric receptor (CH28-3) by flow cytometry using anti-CD4 antibodies (Leu3A). The ability of CH28-3 to provide co-stimulation in primary human CD8+ T cells was tested in an in vitro assay. Activation of T cells by stimulation of the TCR with anti-CD3 requires the presence of APCs (ref.), for example normal peripheral blood mononuclear cells (PBMC) which have been rendered incapable of cell division by irradiation or treatment with DNA synthesis inhibitors such as mitomycin C. To test for co-stimulation by the CD4/CD28 chimeric receptor, anti-CD4 antibodies were used in place of PBMC in experiments measuring proliferation (^{3}H-TdR incorporation), CD69 induction (FACS), and cytokine production (ELISA). T cells are treated with unlabeled anti-CD3 antibodies alone or anti-CD3 antibodies plus anti-CD4 antibodies (to bind the CH28-3 receptor) for 30 min. at 4° C. The cells are washed extensively to remove unbound antibodies and incubated in 24-well tissue culture plates in wells coated with goat anti-mouse IgG at 37° C. Crosslinking of cell surface receptors in this way is sufficient to induce signal transduction. First, induction of CD69 expression will be determined by flow cytometry. T cells treated as described above will be collected after 18 h incubation an analyzed for CD69 expression by staining with a fluorochrome-labeled antibody to CD69. It is expected that at suboptimal doses of anti-CD3, little to no induction of CD69 expression will be observed. Under these conditions the additional treatment of cells with anti-CD4 antibodies, binding to the CH28-3 receptor should restore the induction of CD69 expression. Stimulation with PMA serves as a positive control. Another important feature of T cell activation is the production of cytokines, for example IL-2, IL-4, GM-CSF, and γ-IFN. The ability of CH-28-3 expressing T cells to secrete cytokines in response to anti-CD3 stimulation with or without CH28-3-mediated co-stimulation is determined using the experimental system described above. Second, cytokine production is determined by harvesting the culture supernatant from the cells after 48 h and measuring cytokine levels by ELISA (Quantikine kits, R & D Systems, Minneapolis, Minn.). As a control for these experiments T cells are cultured with soluble anti-CD3 and APCs. Thus, in these experiments, co-stimulation by binding of the CH28-3 chimeric receptor should result in increased levels of cytokine production. The effect of co-stimulation may vary for different cytokines. Third, the ability of CH28-3 bearing T cells to proliferate is determined by measuring incorporation of ^{3}H-thymidine between 54–72 h after stimulation. The T cells are stimulated as described above with anti-CD3 antibodies with or without anti-CD4 co-stimulation in 96-well tissue culture plates, with each test re-produced in triplicate. after 54 h in culture, 3H-thymidine is added, and the cells are harvested after 18 h. DNA from the cells is harvested onto glass fiber filters and incorporation of tritium measured by scintillation counting as a measure of DNA synthesis. Co-stimulation by CH28-3 is expected to support T cell proliferation at suboptimal doses of anti-CD3, where little to no response is expected with anti-CD3 alone. Similar experiments are carried out using T cells expressing a zeta-based chimeric receptor in addition to CH28-3. For example, using T cells expressing F15 (SAb-zeta) the same experiments are performed, but where anti-human IgG Fc antibodies are substituted for anti-CD3 to provide primary stimulation via SAb-zeta.

EXAMPLE 3

Increased growth and transduction efficiency of T cells co-stimulated via CH28-3

It has been reported that co-stimulation by anti-CD28 antibodies enhances the growth rate and cloning efficiency

of human CD8+ T cells grown in vitro (Riddell and Greenberg, supra), and may augment their capacity for autocrine IL-2 production. Since the use of T cells expressing chimeric receptors for in vivo therapy may require the generation of large numbers (>10^9) transduced T cells in vitro prior to infusion, developing a means of increasing the efficiency of this process would be of obvious benefit. First, if cells could be expanded in vitro more rapidly, the initial number of donor cells could be reduced, and the time required to generate the necessary number of cells may be shortened. Second, since retrovirus transduction requires that cells be in an active growth phase for successful integration of provirus, a means of driving a greater proportion of cells into the growth phase of the cell cycle at any given time might increase the efficiency of retroviral transduction. The ability of CD28-based chimeric receptors to perform these functions is tested by introducing the CH28-3 chimeric receptor into human primary CD8+ T cells, and comparing the growth rates of these T cells when stimulated weekly with anti-CD3 and APCs or anti-CD3 plus anti-CD4 plus APCs in the presence of IL-2. The growth rate is determined by determining the number of viable cells present in the cultures and plotting cell number vs. time. The ability to augment retrovirus transduction efficiency is tested by exposing T cells with and without CH28-3 to retrovirus containing the gene for a zeta-based chimeric receptor (e.g., F15) and determining by FACS analysis the percentage of cells expressing the zeta receptor. The expectation is that T cells with CH28-3, when co-stimulated with anti-CD4 antibodies will achieve higher numbers of cells in a shorter period of time than cells stimulated with anti-CD3 alone, and that CH28-3 T cells co-stimulated with anti-CD4 will be transduced with a higher efficiency that those cells stimulated with anti-CD3 alone.

EXAMPLE 4

Construction of a CD4/CD2 Chimeric Receptor

CD4/CD2 chimeric receptors were constructed as described above in Example 1. Oligonucleotides used to amplify CD2 DNA from the cDNA library were oligonucleotides 1 and 2 (SEQ ID NO:1 and SEQ ID NO:2) (FIG. 2). CH2-1 consisted of the extracellular and transmembrane domains of human CD4 fused to the cytoplasmic domain of human CD2; CD2-2 consisted of the extracellular domain of CD4 fused to the transmembrane and cytoplasmic domains of CD2; and CH2-3 consisted of the extracellular domain of CD4 fused to a portion of the extracellular domain plus the entire transmembrane and cytoplasmic domains of CD2. The portion of the extracellular domain of CD2 contains a cysteine residue (position 203) which is important for the formation of homodimers of CD2 at the cell surface. Oligonucleotides used to generate the fusions were as described in FIG. 2. CH2-1: oligonucleotide 7 (SEQ ID NO:7); CH2-2: oligonucleotide 8 ((SEQ ID NO:8); CH2-3: oligonucleotide 9 (SEQ ID NO:9). These constructs are depicted in FIG. 1A.

The ability of the three CD4-CD2 chimeric receptors to synergize with the native TCR in the manner of the native CD2 receptor was evaluated by measuring IL-2 secretion. Three representative clones stably expressing the chimeric constructs CH2-1, CH2-2 and CH2-3, respectively, were subject to stimulation via the native CD3/TCR with Mab OKT3 (Ortho) in the presence or absence of native CD2 stimulation with the anti-CD Mab OKT11 (Ortho). At least a 3-fold augmentation of IL-2 production was observed with CD2 co-stimulation as compared to stimulation via the TCR alone. Antibodies directed against the extracellular domain of either of the chimeric CD2 constructs (OKT4) could elicit the same response as that obtained upon stimulation of the native CD2. Specifically, OKT4 in combination with the TCR antibody OKT3 led to augmentation of the TCR signal by at least 3-fold. A similar co-stimulatory response was obtained upon stimulation of all three CD2 chimeric proteins, and was similar to that obtained from the native CD2 receptor. These results indicate that the cytoplasmic domain of CD2 is sufficient for the synergistic effect of CD2 on TCR-mediated T cell activation.

EXAMPLE 5

Protection from anergy by antibody-driven co-stimulation

Stimulation of T cells through the TCR, for example by anti-CD3 antibodies, in the absence of a co-stimulatory signal, such as one delivered via the cytoplasmic domain of CD28, results in a state of specific non-responsiveness called anergy. Anergic T cells may proliferate initially in response to the anergizing signal, but are rendered non-responsive to a second signal given through the TCR (Johnson and Jenkins, supra). Co-stimulation of the TCR and the CD28 receptor protect cells from entering a state of anergy. The ability of the CD28-based chimeric receptors such as CD4/CD28 to protect T cells from becoming anergic is tested as follows: T cells expressing CH28-3 will be stimulated in vitro with antibodies to CD3 bound to a solid surface (tissue culture wells) in the absence of APCs. In parallel these cells are stimulated in wells coated with both anti-CD3 and anti-CD4 (which stimulate the CD4/CD28 receptor). As a control the T cells are stimulated with anti-CD3 in the presence of APCs as described above. After 7–10 days, the cells are stimulated a second time with anti-CD3 and APCs in each case. T cells originally cultured with anti-CD3 alone should fail to proliferate (i.e., incorporate ^{3}H-TdR) in response to anti-CD3 even under optimal conditions. If the stimulation of the CD28 chimeric receptor affords protection from anergy, T cells expressing CH28-3 stimulated with anti-CD3 and anti-CD4 should respond normally to anti-CD3 in the presence of APCs, in the same manner as cells stimulated originally with anti-CD3 in the presence of APCs.

EXAMPLE 6

Function of CH28-3 in Primary human T cells expressing a zeta-based chimeric receptor

Since it has been demonstrated that the CD28 chimeric receptors function in conjunction with zeta-based chimeric receptors (above), this experimental model can also be used with primary T cells expressing both a zeta-based chimeric receptor and CH28-3. Primary human CD8+ T cells expressing a SAb-zeta chimeric receptor (F15, as described in U.S. Pat. No. 5,359,046) are transduced with the CH28-3 chimeric receptor by retrovirus-mediated gene transfer as described above. Cells expressing both chimeric receptors are identified by FACS analysis using anti-human IgG Fc antibodies to detect the SAb-zeta (F15) receptor, and anti-CD4 antibodies to detect CH28-3. These cells are tested for their susceptibility to anergy via CD3/TCR stimulation and F15 stimulation as described above using anti-CD3 antibodies, or anti-Fc antibodies (Caltag Laboratories, So. San Francisco, Calif.) to stimulate the SAb-zeta receptor, in the absence of APCs, together with anti-CD4 antibodies for co-stimulation.

EXAMPLE 7

Protection from anergy induced by Co-stimulation-Deficient Tumor Cells

Both F15 and CH28-3 recognize antigens displayed by 293 cells transfected with the HIV envelope gene. These cells stimulate proliferation and serve as targets for lysis by cytotoxic T cells expressing F15 (Roberts et al., *Blood*, 84(9):2878–2889 (1994)). APCs or target cells which are fixed (e.g., by paraformaldehyde) are not able to provide co-stimulation even though they express the appropriate antigen for recognition by T cells (Johnson and Jenkins, supra, and Otten and Germain *Science* 251:1228–1231 (1991)). The ability of CH28-3 to protect T cells from anergy by exposure to fixed target cells is tested by comparing the responses of T3/F15 T cells with and without CH28-3 to live 293 env cells after prior exposure to fixed 293 env cells. CD8+ F15 T cells are co-cultured with an experimentally determined number of live, mitomycin-treated or paraformaldehyde fixed 293 cells expressing the HIV envelope protein, in the presence of IL-2. After 7–10 days the cells are challenged by re-exposure to live 293 env cells. First, the ability of CD8/F15 cells to proliferate in response to live, mitomycin-treated 293 env cells is determined by thymidine incorporation assay. Second, cytokine production in response to 293 env is measured under the same experimental conditions by ELISA as described above. Third, the antigen-specific cytolytic function of the cells is determined using the JAM assay for target cell lysis (Matzinger, *J. Immunol. Methods* 145:185 (1991)). Briefly, 293 env cells are labeled overnight with 3H-thymidine, and plated in 96-well plates the next day, together with CD8/F15 cells. Lysis of the 293 env cells is determined by counting the amount of radioactive DNA released into the supernatant by the damaged cells in 6 h. The expectation is that CD8/F15 cells without CH28-3 will become anergic when exposed to fixed 293 env cells, due to lack of co-stimulation, and will fail to proliferate, produce cytokines, or kill 293 env targets upon re-exposure. In contrast, CD8/F15 cells which express CH28-3 will receive the necessary co-stimulation from fixed 293 env, and will proliferate, produce cytokines, and kill normally when re-exposed to live 293 env.

EXAMPLE 8

Protection from anergy induced by cell-free virus particles

A potential use of T cells expressing chimeric receptors which recognize HIV antigens is to introduce them into HIV-infected individuals as a form of anti-viral therapy. In vivo the T cells will encounter large amounts of soluble antigen in the patient's serum which may be capable of rendering these anergic to stimulation by their true targets, HIV-infected cells. For example, there is sufficient soluble gp120 shed by virus particles and infected cells in the serum of HIV seropositive individuals to prime CD4+ positive T cells to undergo apoptosis, a programmed cell death mechanism, when they are stimulated by antigen or polyclonal mitogens (Banda et al., *J. Exp. Med.* 176:1099–1106 (1992)). The susceptibility of T cells armed with anti-HIV chimeric receptors to anergy induced by soluble gp120 or cell-free virus particles is tested by incubating CD8/F15 cells, with or without CH28-3, with soluble gp120 (e.g. 50 ng/ml) or with heat-inactivated culture supernatant from HIV-infected T cells which contains various amounts of HIV by p24 content, e.g. 500 pg/ml of p24 gag (Roberts et al., (1994) supra). Treatment of the cells with soluble antigens is in the absence of APCs. After 7–10 days, the cells will be exposed to 293 env cells, and their biological responses (i.e., proliferation, cytokine production, and cytotoxicity) are measured by standard assays as described above. CD8/F15 cells co-expressing CH28-3 are protected from anergy induced by soluble antigens, but those without the CD28-based chimeric receptor are susceptible.

EXAMPLE 9

Enhancement of Anti-tumor immunity in vivo in transgenic mice expressing CH28-3

The murine T cell lymphoma line EL-4 produces nodular tumors when introduced into syngeneic mice (Chen et al., *J. Exp. Med.* supra). EL-4 cells lack expression of the B7 ligand for CD28 and do not elicit protective immunity except by repeated injection of large numbers of irradiated tumor cells (ibid.). In contrast, EL-4 cells transduced to express B7 are unable to form tumors in syngeneic mice. Instead, injection of mice with EL-4/B7 cells causes the regression of existing EL-4 tumors and confers lasting protective immunity against subsequent injections with B7$^-$ EL-4 cells (ibid.). The ability of CD28-based chimeric receptors to augment the immune response to a relatively non-immunogenic tumor is tested in vivo using mice which are transgenic for the expression of CH28-3. CH28-3 DNA is introduced into the genome of C57BL/6 mice, which are syngeneic to EL-4 cells, by micro-injection of the transgenic expression vector pIK.Mcd2(en/dcr)CH28-3. In this vector the CH28-3 chimeric co-receptor is expressed under the control of the murine mammary leukemia virus (MMLV) promoter and a transcriptional control region of the CD2 gene, designated the enhancer-dcr region (Lake et al., *EMBO* 9:3129–3136 (1990)). The CD2 en/dcr transcriptional control region drives expresion of the CD2 gene in lymphocytes, and has been shown to confer high level tissue-specific position-independent expression of the gene when introduced into the germ line of mice (Lang et al. *Nucleic Acids Res.* 19:5851–5856 (1991)). Therefore, integration of the pIK.Mcd2 (en/dcr)CH28-3 vector into the genome of C57BL/6 mice results in expression of the CH28-3 chimeric co-receptor in murine lymphocytes. Mice whose lymphocytes express high levels of the CH28-3 receptor were identified by analyzing blood and lymphoid tissue by FACS with antibodies to human CD4. Such mice will be injected with non-manipulated EL-4 cells or EL-4 cells expressing the HIV envelope protein. The ability of these cells to form tumors in CH28-3 transgenic and normal mice is compared. The expectation is that stimulation of the CH28-3 receptor on transgenic T cells by HIV env expressed on the EL-4 cells will stimulate an immune response to EL-4 cells which will be absent in normal mice. Thus, the CH28-3 transgenic animals should reject the EL-4 cells and show no or greatly reduced formation of tumors. The non-manipulated EL-4 cells should form tumors in both kinds of mice. These experiments demonstrate the ability of CD28-based chimeric receptors to provide co-stimulation in a situation in which antigen-responsive cells are present, but the natural immune response is insufficient due to a lack of co-stimulation.

EXAMPLE 10

Increase functional activity of adoptively transferred cells in vivo

For these experiments a small animal model is used to study the function of primary human T cells expressing

zeta-based and CD28-based chimeric receptors in vivo. Two human disease models are established in SCID mice (Bosman and Carroll, *Annu. Rev. Immunol.* 9:323–350 (1991)): (i) a tumor model using human tumor cells, and (ii) a virus infection model using HIV-infected human PBL (McCune et al., ibid 399–430; Mosier et al., *Nature* 335:256–259 (1988)). For example, the human B lymphoblastoid cell line Raji produces lethal tumors when engrafted into SCID mice (Malkovska et al., *Cancer Res.* 52:5610–5616 (1992)). Raji cells expressing HIV envelope also produce tumors in SCID mice. Raji env cells provide a convenient model for testing human T cells bearing chimeric receptors which recognize HIV envelope determinants such as CD4-zeta, Sab-zeta (F15), and CD4-CD28 (CH28-3). T cells expressing a zeta-based UR with or without CH28-3 are introduced into SCID mice together with antigen-bearing tumor cells. The longevity of cells introduced with tumor cells are compared to the survival of cells in mice with out tumor cells, by isolating the human T cells from the blood and lymphoid tissue of SCID mice at sequential time intervals. The number of human T cells present is determined, as well as expression of the chimeric receptors, and the ability to proliferate and to kill appropriate target cells in vitro. One expectation would be that in the presence of tumor cells which fail to provide normal co-stimulation the lifespan and biological activity of the chimeric receptor T cells will be prolonged if those cells possess a CD28-based chimeric receptor in addition to a zeta-based receptor both of which recognize the same target cells. The mice can also be analyzed for tumor progression, metastases, and the like. Similarly, SCID mice engrafted with HIV-infected human PBL are used to assess the functional activity of chimeric receptor bearing T cells in an in vivo model of HIV infection. T cells expressing zeta-based chimeric receptors with and without a CD28-based chimeric receptor are introduced into SCID mice harboring HIV-infected human PBL. The longevity of the cells and biological activity against HIV is measured as described above, along with viral load in the mice, by p24 assay (Coulter Immunology, Hialeah, Fla.) and persistence of CD4 T cells.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 16

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CACCACCAGC ACCCAAAATG GCTGCACCGG GGTGGA 36

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TAGGGGACTT GGACAAAGTG GCTGCACCGG GGTGGA 36

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCTCTGTTTT TTCCTTTTGA CACAGAAGAA GATGCC 36

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCCAATGATG AGATAGATTG GCTGCACCGG GGTGGA 36

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

-continued

(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ACCTTTCTCT GGACAGCTTG GCTGCACCGG GGTGGA 36

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCTGCTCCTC TTACTCCTCC GGCACCTGAC ACAGAA 36

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCTGCTCCTC TTACTCCTGA AGAAGATGCC TAGCCC 36

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AATATTGAAT TCCGAGCTTC GAGCCAA 27

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AATATTGGTT ACCAGTGGCT GTTGCACAGG G 31

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

-continued

CGCCCCCCAG CACAATCAGG GCCATTGCGC CCCCCGCCGC CGCTGGCCGG CAC 53

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTGCGCTCCT GCTGAACTTC ACTCTATTTG CAAACACGTC TTCGGTTCCT 50

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CATCCAGCAG GTAGCAGAGT TTGGGTGCGC CCCCCGCCGC TGGCCGGCAC 50

What is claimed is:

1. A DNA encoding a chimeric membrane-bound protein, said protein comprising in the N-terminal to C-terminal direction:

a signal sequence;

an extracellular binding domain of a surface membrane or secreted protein that binds specifically to at least one ligand;

a transmembrane domain; and

a cytoplasmic domain of CD2 or CD28;

wherein said extracellular domain is not obtained from CD2 or CD28, and when said DNA is placed in a selected host cell under conditions suitable for expression, said chimeric membrane-bound protein is expressed and co-stimulates effector function signaling in said host cell upon binding of a ligand to the extracellular domain.

2. The DNA according to claim 1 wherein said extracellular domain is from a cell differentiation antigen.

3. The DNA according to claim 2 wherein said extracellular domain is from CD4.

4. The DNA according to claim 2 wherein said extracellular domain is from CD8.

5. The DNA according to claim 1 wherein said extracellular domain is from an antibody or single-chain antibody or portions or modifications thereof containing ligand binding activity.

6. The DNA according to claim 5 wherein said antibody or single-chain antibody recognizes an antigen selected from the group consisting of viral antigens and tumor cell associated antigens.

7. The DNA according to claim 1 wherein said DNA encoding the cytoplasmic domain further comprises a DNA encoding a cytoplasmic effector function signaling domain that transduces an effector function signal in a host cell upon binding of a ligand to the extracellular domain.

8. An expression cassette comprising a transcriptional initiation region, a DNA according to claim 1 under the transcriptional control of said transcriptional initiation region and a transcriptional termination region.

9. A cell comprising a DNA according to claim 1.

10. A cell comprising a DNA that encodes a chimeric effector function receptor comprising an extracellular ligand-binding domain, a transmembrane domain and a cytoplasmic effector function signaling domain, and a second DNA according to claim 1.

11. A DNA encoding a hybrid chimeric membrane-bound protein, said protein comprising in the N-terminal to C-terminal direction;

a signal sequence;

an extracellular binding domain of a surface membrane or secreted protein that binds specifically to at least one ligand;

a transmembrane domain;

a cytoplasmic domain of CD2 or CD28; and

a cytoplasmic effector function signaling domain;

wherein said extracellular domain is not obtained from CD2 or CD28, and when said DNA is placed in a selected host cell under conditions suitable for expression, said hybrid chimeric membrane-bound protein is expressed and initiates an effector function signal and a co-stimulatory effector function signal upon binding of a ligand to said extracellular domain.

12. The DNA of claim 11 wherein said cytoplasmic effector function signaling domain is selected from the group consisting of the cytoplasmic effector function signaling domains CD3 zeta chain, the CD3 eta chain, the CD3 gamma chain, the CD3 delta chain, the CD3 epsilon chain, the beta chain of the FceR1 receptor, the gamma chain of the FceR1 receptor, the B29 (Ig beta) chain of the B cell receptor, and a tyrosine kinase.

13. An expression cassette comprising a transcriptional initiation region, a DNA according to claim 11 under the transcriptional control of said transcriptional initiation region and a transcriptional termination region.

14. A cell comprising a DNA according to claim 11.

15. A cell comprising a DNA that encodes a chimeric effector function receptor comprising an extracellular ligand-binding domain, a transmembrane domain and a cytoplasmic effector function signaling domain, and a second DNA according to claim 11.

16. A DNA encoding a hybrid chimeric membrane-bound protein, said protein comprising in the N-terminal to C-terminal direction;

a signal sequence;

an extracellular binding domain of a surface membrane or secreted protein that binds specifically to at least one ligand;

a transmembrane domain;

a cytoplasmic effector function signaling domain; and

a cytoplasmic domain of CD2 or CD28;

wherein said extracellular domain is not obtained from CD2 or CD28, and when said DNA is placed in a selected host cell under conditions suitable for expression, said hybrid chimeric membrane-bound protein initiates an effector function signal and a co-stimulatory effector function signal upon binding of a ligand to said extracellular domain.

17. The DNA of claim 16 wherein said cytoplasmic effector function signaling domain is selected from the group consisting of the cytoplasmic effector function signaling domains of the CD3 zeta chain, the CD3 eta chain, the CD3 gamma chain, the CD delta chain, the CD3 epsilon chain, the beta chain of the FcγR1 receptor, the B29 (Ig beta) chain of the B cell receptor, and a tyrosine kinase.

18. An expression cassette comprising a transcriptional initiation region, a DNA according to claim 16 under the transcriptional control of said transcriptional initiation region and a transcriptional termination region.

19. A cell comprising a DNA according to claim 16.

20. A cell comprising a DNA that encodes a chimeric effector function receptor comprising an extracellular ligand-binding domain, a transmembrane domain and a cytopasmic effector function signaling domain, and a second DNA according to claim 16.

21. The cell according to any one of claims 9, 10, 14, 15, 17 and 20 wherein said cell is a mammalian cell.

22. The cell according to any one of claims 9, 10, 14, 15, 17 and 20 wherein said cell is a human cell.

23. A chimeric co-stimulatory receptor protein comprising in the N-terminal to C-terminal direction;

an extracellular ligand binding domain that binds specifically to at least one ligand;

a transmembrane domain; and

a cytoplasmic co-stimulatory signaling domain of CD2 or CD28;

wherein said extracellular domain is not obtained from CD2 or CD28, and when said chimeric co-stimulatory protein is expressed as a membrane-bound receptor in a host cell under conditions suitable for expression said membrane-bound receptor initiates a co-stimulatory effector function signal in said host cell upon binding of a ligand to the extracellular domain.

24. A hybrid chimeric co-stimulatory receptor protein comprising in the N-terminal to C-terminal direction;

an extracellular ligand binding domain that binds specifically to at least one ligand;

a transmembrane domain;

a cytoplasmic co-stimulatory signaling domain of CD2 or CD28; and

a cytoplasmic effector function signaling domain,

wherein said extracellular domain is not obtained from CD2 or CD28, and when said hybrid chimeric co-stimulatory protein is expressed as a membrane-bound receptor in a host cell under conditions suitable for expression, said membrane-bound receptor initiates an effector function signal and a co-stimulatory effector function signal in said host cell upon binding of a ligand to the extracellular domain.

25. A hybrid chimeric co-stimulatory receptor protein comprising in the N-terminal to C-terminal direction;

an extracellular ligand binding domain that binds specifically to at least one ligand;

a transmembrane domain;

a cytoplasmic effector function signaling domain; and

a cytoplasmic co-stimulatory signaling domain of CD2 or CD28;

wherein said extracellular domain is not obtained from CD2 or CD28, and when said hybrid chimeric co-stimulatory protein is expressed as a membrane-bound receptor in a host cell under conditions suitable for expression said membrane-bound receptor initiates an effector function signal and a co-stimulatory effector function signal in said host cell upon binding of a ligand to the extracellular domain.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,712,149
DATED : January 27, 1998
INVENTOR(S) : Margo R. Roberts

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 2, line 24, delete "it" and insert therefor --It--.

Col. 3, line 61, delete "257;217" and insert therefor --257:217--.

Col. 4, line 15, delete "CD21" and insert therefor --CD2--.

Col. 5, line 66, delete "invivo" and insert therefor --in vivo--.

Col. 14, line 46, delete "Mycobactium" and insert therefor --Mycobacterium--.

Col. 17, line 41, delete "GD18" and insert therefor --G418--.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,712,149

DATED : January 27, 1998

INVENTOR(S) : Margo R. Roberts

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 20, line 23, delete "an" and insert therefor --and--; and

line 49, delete "re-produced" and insert --reproduced--;

delete "after" and insert therefor --After--.

Col. 24, line 35, delete "expresion" and insert --expression--.

Col. 32, line 59, after "domains" insert --of the--.

Signed and Sealed this

Ninth Day of June, 1998

Attest:

Attesting Officer

BRUCE LEHMAN

Commissioner of Patents and Trademarks

US005504000A

United States Patent [19]

Littman et al.

[11] Patent Number: 5,504,000

[45] Date of Patent: Apr. 2, 1996

[54] **CHIMERIC PROTEIN TYROSINE KINASES**

[75] Inventors: **Dan Littman**; **Hua Xu**, both of San Francisco, Calif.

[73] Assignee: **Regents of the University of California**, Oakland, Calif.

[21] Appl. No.: **459,170**

[22] Filed: **Jun. 2, 1995**

Related U.S. Application Data

[62] Division of Ser. No. 112.912, Aug. 27, 1993, Pat. No. 5,439,819.

[51] **Int. Cl.**[6] **C12N 9/12**; C12N 5/00; C12P 21/06; C07H 19/00

[52] **U.S. Cl.** **435/194**; 435/69.1; 435/69.7; 435/240.2; 530/350; 536/22.1; 536/23.1; 536/23.2; 536/23.4; 536/23.5

[58] **Field of Search** 435/69.1, 69.7, 435/194, 240.2; 530/350; 536/22.1, 23.1, 23.2, 23.4, 23.5

[56] **References Cited**

PUBLICATIONS

Glaichenhaus et al. "Requirement for Association of p56 ^{lck} . . . " Cell 64:511–520 (1991).

Turner et al. "Unteraction of the Unique N–Terminal . . . " Cell 60:755–765 (1990).

Primary Examiner—Robert A. Wax
Assistant Examiner—Hyosuk Kim
Attorney, Agent, or Firm—Townsend and Townsend and Crew

[57] **ABSTRACT**

The present invention provides chimeric proteins containing extracellular and transmembrane domains of CD4 and protein tyrosine kinases of the src family. Also provided are DNA molecules encoding the proteins of the present invention and cells containing such DNA molecules. The proteins and cells of the present invention may be employed in methods for identifying drugs that block T cell activation and for identifying low level self-antigens.

5 Claims, 7 Drawing Sheets

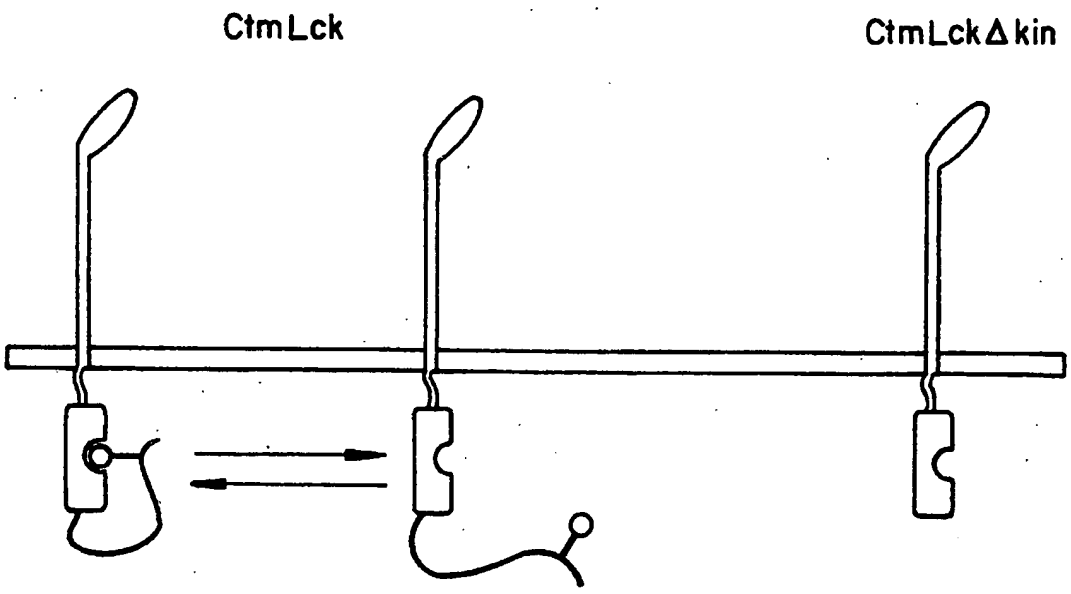

CtmLck
CtmLckΔkin

FIG. 1.

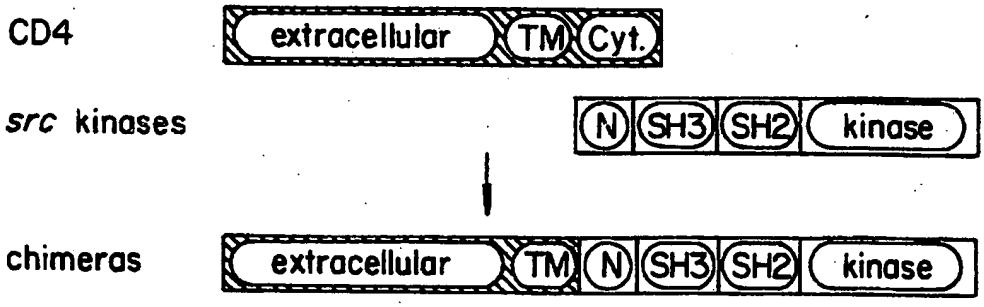
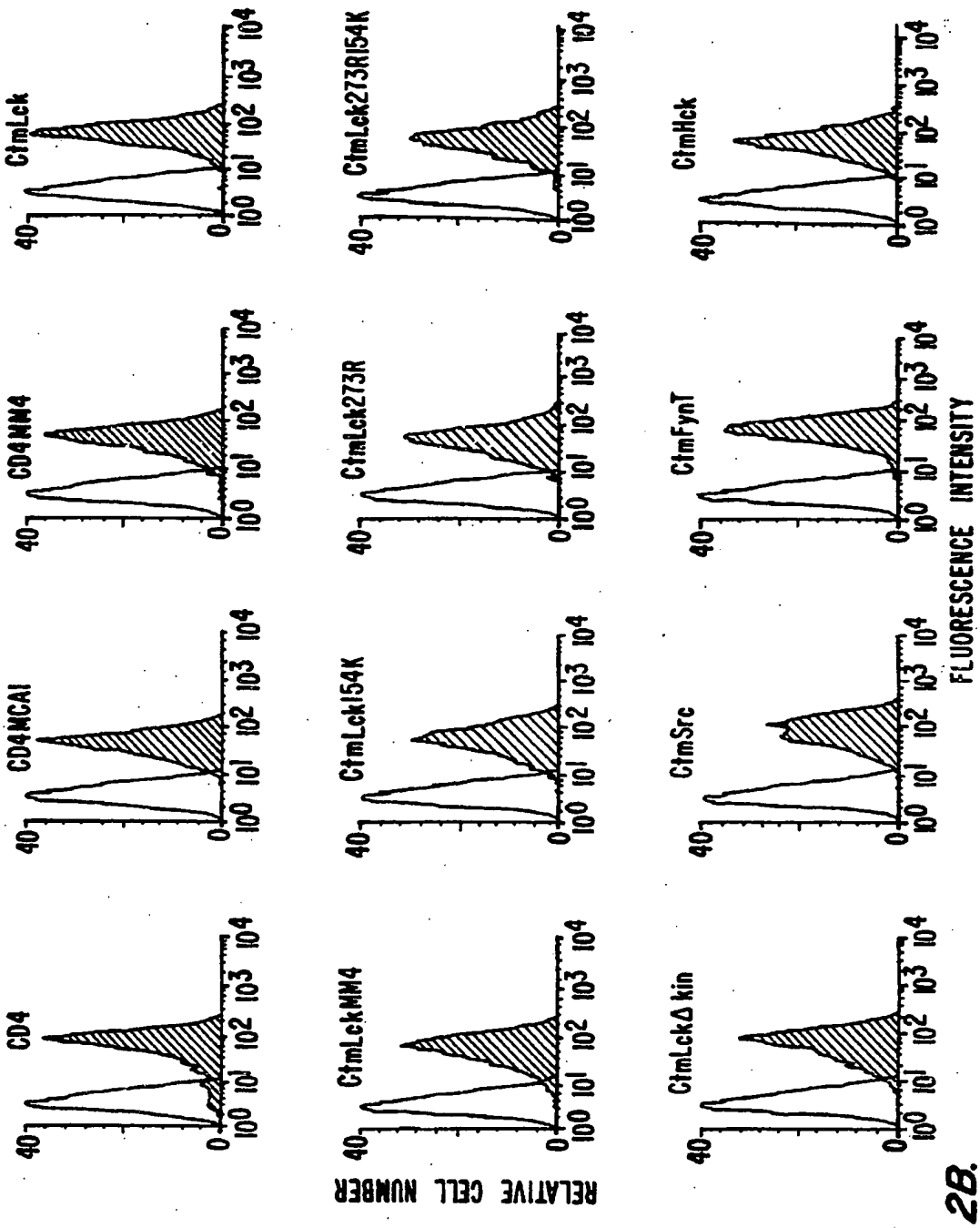

CD4
extracellular
TM
Cyt.
src kinases
N
SH3
SH2
kinase
chimeras
extracellular
TM
N
SH3
SH2
kinase

FIG. 2A.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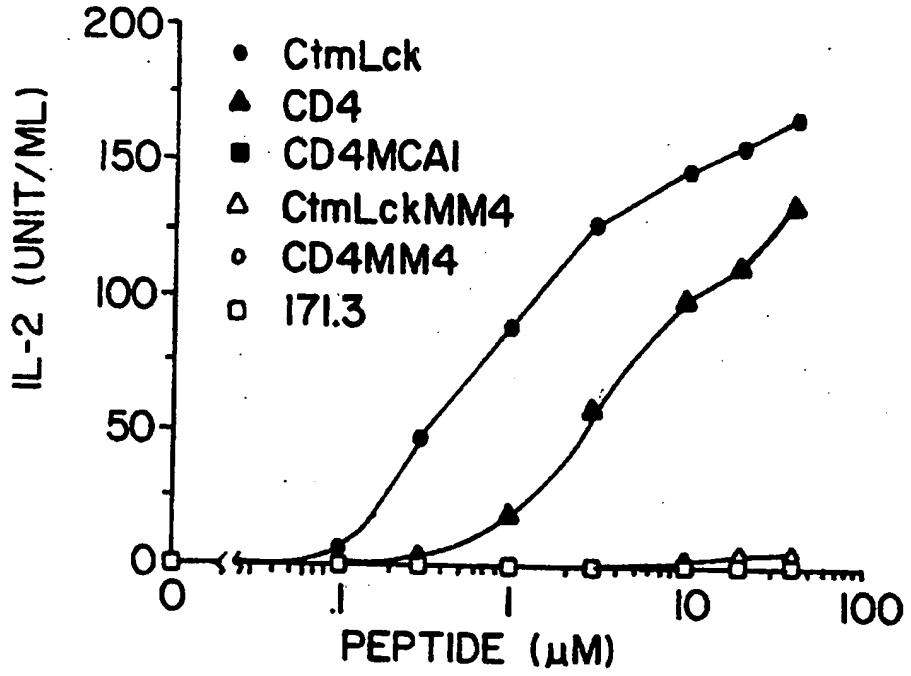
CD4MM4
CtmLck
CtmLckMM4
CtmLck154K
CtmLck273R
CtmLck273R154K
CtmLckΔkin
CtmSrc
CtmFynT
CtmHck
40
0
10^0 10^1 10^2 10^3 10^4
RELATIVE CELL NUMBER
FLUORESCENCE INTENSITY

FIG. 2B.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150
100
50
0
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CD4
CD4MCAI
CtmLckMM4
CD4MM4
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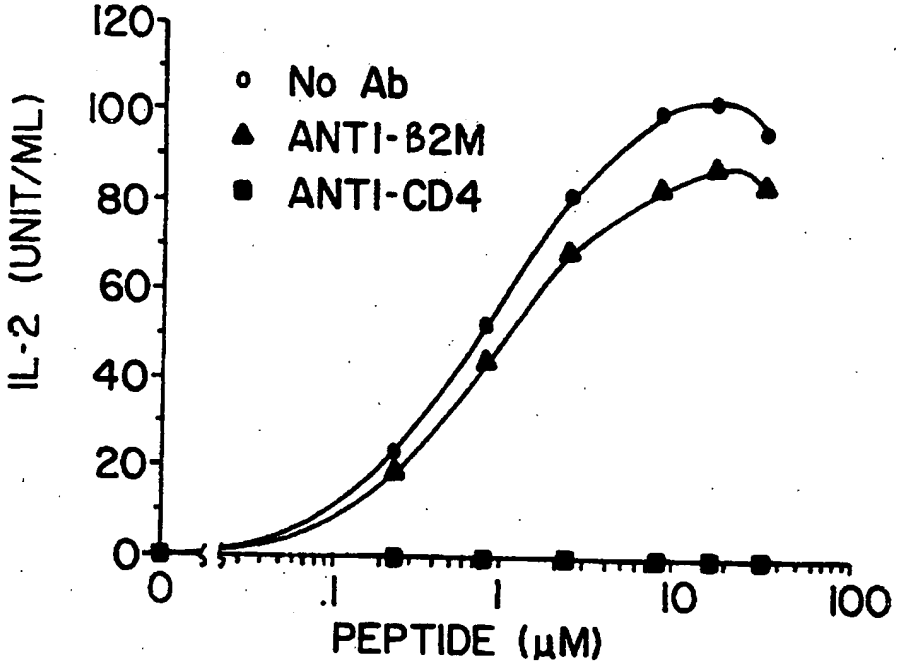
0
.1
1
10
100
PEPTIDE (μM)

FIG. 3A.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60
40
20
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ANTI-β2M
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1
10
100
PEPTIDE (μM)

FIG. 3B.

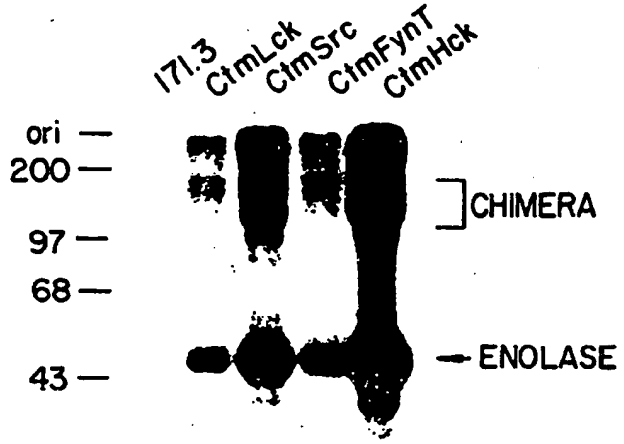


FIG. 4A.

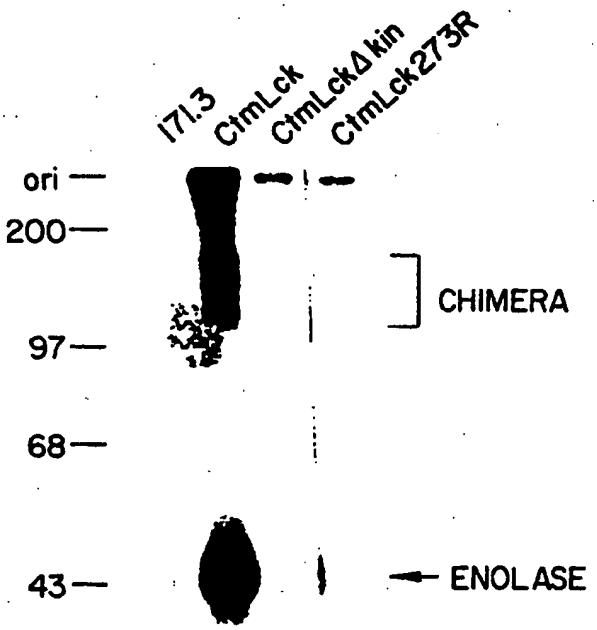


FIG. 5A.

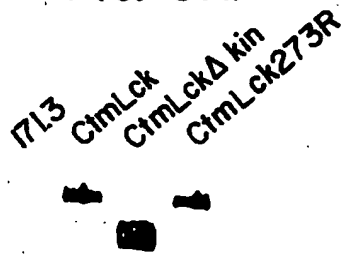


FIG. 5B.

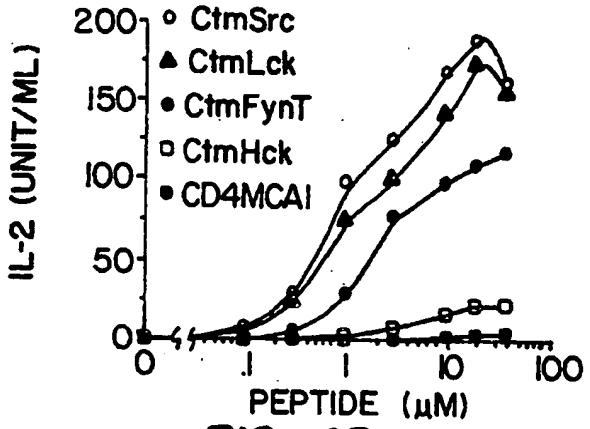


FIG. 4B.

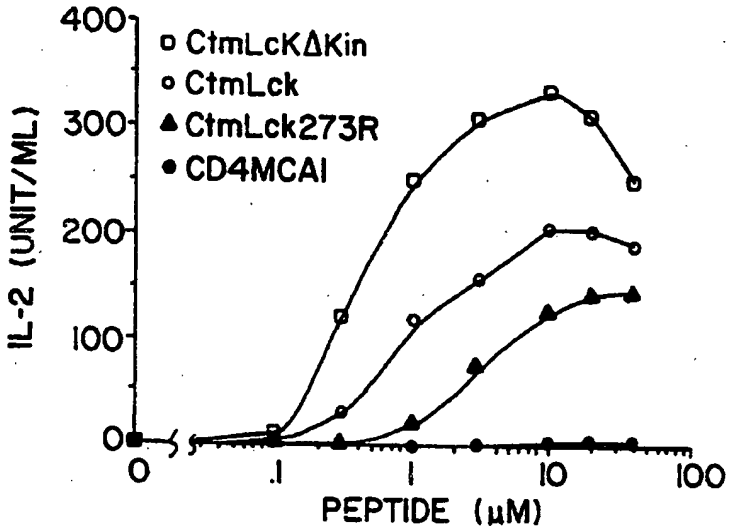


FIG. 5C.

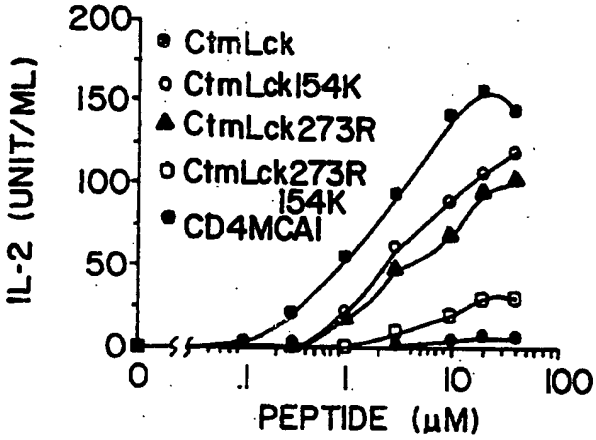


FIG. 7C.

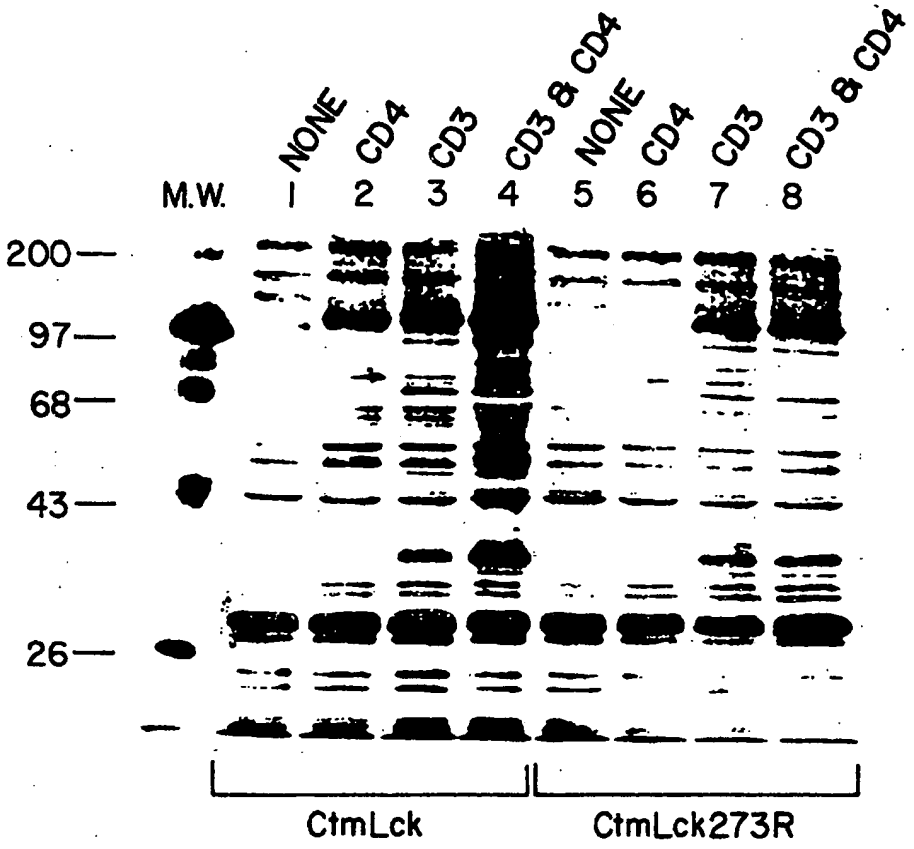
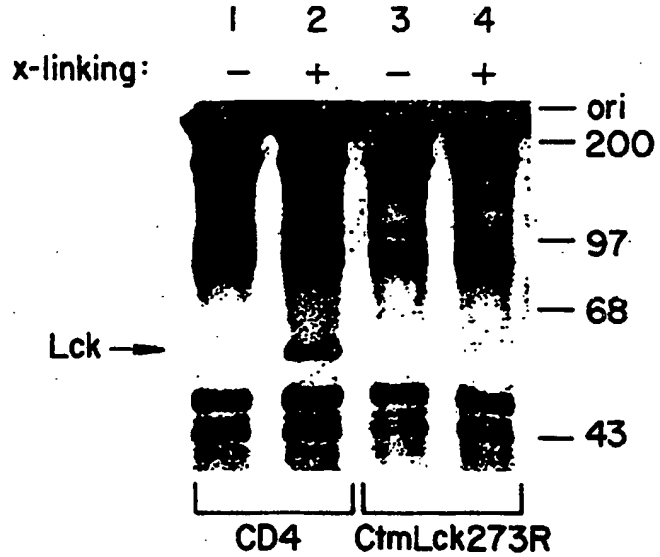

NONE
CD4
CD3
CD3 & CD4
NONE
CD4
CD3
CD3 & CD4
M.W.
1 2 3 4 5 6 7 8
200
97
68
43
26
CtmLck
CtmLck273R

FIG. 6A.


1 2 3 4
x-linking: − + − +
ori
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Lck
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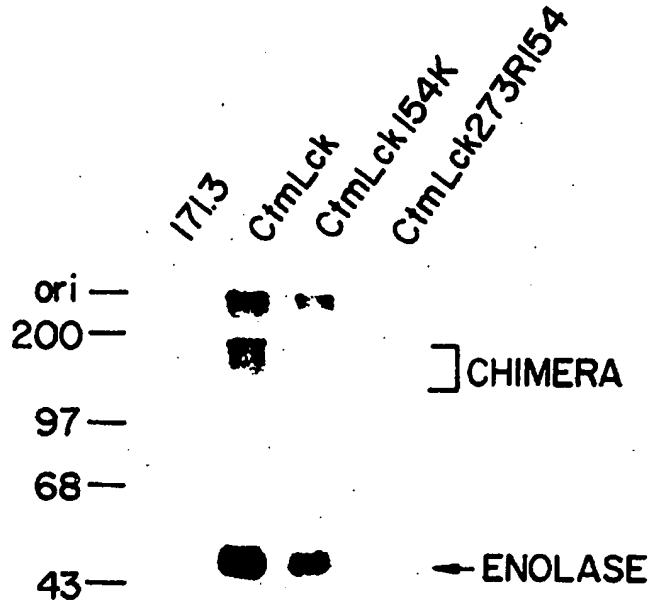
CD4
CtmLck273R

FIG. 6B.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CtmLck154K
CtmLck273R154
ori
200
97
68
43
CHIMERA
ENOLASE

FIG. 7A.

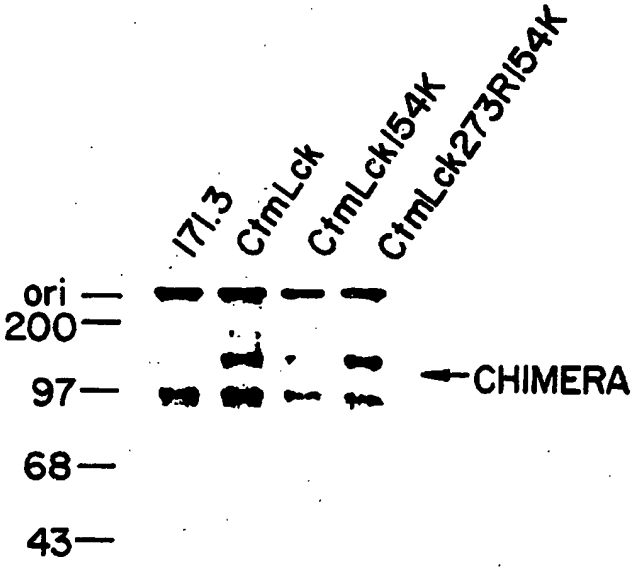

171.3
CtmLck
CtmLck154K
CtmLck273R154K
ori
200
97
68
43
CHIMERA

FIG. 7B.

CHIMERIC PROTEIN TYROSINE KINASES

This is a division of application Ser. No. 08/112,912 filed Aug. 27, 1993, now U.S. Pat. No. 5,439,812.

BACKGROUND OF THE INVENTION

The present invention relates generally to chimeric proteins comprising the extracellular and transmembrane portions of CD4 molecules and an src protein tyrosine kinase. Such CD4 chimeric proteins may amplify the signal produced by T lymphocyte stimulation. These CD4 chimeric proteins may be employed to identify drugs that block $CD4^+$ T lymphocyte activation. The CD4 chimeric proteins are also useful for identifying self antigens that may mediate autoimmune diseases. Also provided are CD8 chimeric proteins having an src tyrosine kinase linked to the extracellular and transmembrane portions of a CD8 molecule. The CD8 chimeric proteins may be used to screen for MHC Class I restricted antigens. Both the CD4 and CD8 chimeric proteins may be employed in gene therapy treatments to enhance in vivo immunological response to specific antigens.

Cytoplasmic protein tyrosine kinases (PTK's) of the src family have important roles in signal transduction processes in multiple cell types (Bolen et al., *Adv. Can. Res.*, 57:103–149 (1991)). Members of this family share several features: they are attached to cellular membranes through a myristylated N-terminus, they have unique N-terminal domains, and they have homologous SH3, SH2, and catalytic domains (FIG. 1A). Similar SH2 and SH3 domains are found in a wide variety of molecules involved in signal transduction (Koch et al., *Science*, 252:668–674 (1991)). The SH2 domains interact specifically with various proteins containing phosphotyrosine residues, whereas SH3 regions bind guanine nucleotide releasing factors, potentially linking the PTK's to the ras signaling pathway (Feig, *Science*, 260:767–768 (1993)). Multiple src family molecules are expressed in most cells and ablation of individual genes has resulted in developmental defects of variable severity (Soriano et al., *Cell*, 64:693–702 (1991); Molina et al., *Nature*, 357:161–164 (1992); Appleby et al., *Cell*, 70:751–763 (1992); Stein et al., *Cell*, 70:741–750 (1992)). Some functions may be carried out by any one of several src family members, whereas others may only be fulfilled by a single one of these molecules.

Early activation events in T lymphocytes require the triggering of a tyrosine phosphorylation pathway that appears to involve one or more of these molecules (Weiss, *Cell*, 73:209–212 (1993)). A limited number of these kinases, Lck, Fyn, and Yes, are expressed in T cells. Of these, the best-characterized is the lymphocyte-specific tyrosine kinase, $P56^{lck}$ (Lck), whose unique N-terminal domain interacts with the cytoplasmic tails of the CD4 and CD8 glycoproteins. These are molecules that bind to surface MHC class II and class I molecules, respectively, and participate with the T cell antigen receptor (TCR) in early events of T cell activation (Rudd et al., *Proc. Natl. Acad. Sci. USA*, 85:5190–5194 (1988); Veillette et al., *Cell*, 55:301–308 (1988); Shaw et al., *Cell*, 59:627–636 (1989); Turner et al., *Cell*, 60:755–765 (1990); Shaw et al., *Mol. Cell. Biol.*, 10:1853–1862 (1990)). The interaction of Lck with CD4 and CD8 is restricted to this member of the src family and is required for effective antigen-specific responses of several different T cell hybridomas (Zamoyska et al., *Nature*, 342:278–281 (1989); Glaichenhaus et al., *Cell*, 64:511–520 (1991)).

Lck apparently has multiple functions that are essential in T cell development and activation. Inactivation of the Lck gene in mice results in early arrest of thymocyte maturation, prior to cell surface expression of CD4, CD8, and the T cell receptor, suggesting that Lck has a critical function early in T cell development that is independent of these cell surface molecules (Molina et al., *Nature*, 357:161–164 (1992)). In the human T cell leukemic line, Jurkat, absence of Lck results in loss of activation in response to anti-TCR antibodies (Straus and Weiss, *Cell*, 70:585–593 (1992)). The related PTK's present in developing thymocytes and in Jurkat cells appear unable to substitute for Lck. This may be due to a requirement for Lck to associate with cell surface molecules other than CD4 and CD8 that are involved in early development and in TCR-mediated signaling.

Activation of T lymphocytes upon their encounter with MHC-bound peptide antigens is mediated through a complex machinery associated with the T cell antigen receptor (TCR). The clonally-restricted TCR provides specificity for antigen, while associated non-polymorphic polypeptides are involved in the signal transduction process (Irving and Weiss, *Cell*, 64:891–901 (1991); Romeo et al., *Cell*, 68:889–897 (1992); Letourneur and Klausner, *Science*, 255:79–82 (1992); Wegener et al., *Cell*, 68:83–95 (1992)).

In addition, the MHC-binding co-receptor molecules, CD4 and CD8 , are required for initiating signals, both during thymocyte development and in the activation of mature T cells (Fung-Leung et al., *Cell*, 65:443–449 (1991); Rahemtulla et al., *Nature*, 353:180–184 (1991); Killeen et al., *EMBO J.*, 12:1547–1553 (1993)). Apparently, signaling requires coordinate recognition of MHC by the co-receptors and by the TCR. This is achieved by binding of CD4 and CD8 to membrane-proximal domains of class II or class I molecules, respectively, while the TCR binds to the peptide-containing surface (Salter et al., *Nature*, 345:41–46 (1990); Aldrich et al., *Nature*, 352:718–721 (1991); Ingold et al., *Nature*, 352:721–723 (1991); Killeen et al., *J. Exp. Med.*, 176:89–97 (1992); Glaichenhaus et al., *Cell*, 64:511–520 (1991); Konig et al., *Nature*, 356:796–798 (1992)).

The signaling cascade that follows engagement of the T cell receptor by antigen is dependent on the activity of cytoplasmic tyrosine kinases (Klausner and Samelson, *Cell*, 64:875–878 (1991)). Ligation of the TCR results in rapid phosphorylation of intracellular proteins on tyrosine residues (June et al., *J. Immunol.*, 144:1591–1599 (1990)). Inhibitors of PTK function block the early signaling events, notably the phosphorylation and activation of phospholipase Cγ1, a key enzyme involved in the generation of second messengers that regulate intracellular free calcium concentration and the activity of protein kinase C (Mustelin et al., *Science*, 247:1584–1587 (1990)).

Several tyrosine kinases have been implicated in the initiation of the T cell signaling pathway. The lymphoid-specific cytoplasmic PTK, Lck, apparently is a key component in this process: its absence prevents TCR-mediated activation of Jurkat cells (Straus and Weiss, *Cell*, 70:585–593 (1992)), and PLC-γ1 has been co-precipitated with Lck following activation (Weber et al., *J. Exp. Med.*, 176:373–379 (1992)).

A second src family PTK, Fyn-T, has also been implicated in TCR-mediated activation because it is expressed in a T cell-specific manner. Fyn-T is associated with TCR proteins in cell lysates and its level of expression correlates with the magnitude of thymocyte stimulation. A third kinase that appears to have a role in signaling is ZAP-70, a cytoplasmic PTK that is tightly associated with the TCR-ζ chain in activated T cells.

Blocking T cell function is desirable in many instances. For example, blocking T cell activation may provide a means of treating and preventing autoimmune diseases, such as systemic lupus erythematosis, rheumatoid arthritis, Sjogren's syndrome, and the like. Interfering with intracellular signal transduction following antigenic stimulation of T cells could provide a means for reducing excessive inflammation and alleviating many clinical illnesses.

Enhancement of immunological response is desirable in other clinical illnesses. For example, malignancy often impairs immune responses. In these patients, it is desirable to enhance the immune response to help fight infections as well as for primary treatment of the underlying malignancy.

No convenient means have been available to identify drugs that block T cell function at the level of protein tyrosine kinase activity. A means to identify such drugs would provide a marked advance in the art of pharmaceutical development. With such a method, skilled artisans could quickly identify promising compounds for clinical use. Quite surprisingly, the present invention fulfills these and other related needs.

SUMMARY OF THE INVENTION

The present invention provides chimeric proteins comprising a CD4 extracellular domain, a CD4 transmembrane domain, and an src family protein tyrosine kinase. These chimeric proteins may amplify the response of T cells expressing the proteins in response to immunogenic stimuli, such as antigenic stimulation and antibody cross-linking. Generally, the src protein tyrosine kinase will be a human or murine protein tyrosine kinase. Examples of such kinases include $p56^{lck}$, c-SRC Fyn-T or Hck mutations of these tyrosine kinases or fusion proteins having segments of more than one tyrosine kinase. DNA molecules encoding these chimeric proteins and cells transfected with such DNA are also provided.

The CD4 chimeric proteins of the present invention may be employed to determine whether a drug is capable of blocking T lymphocyte activation. The methods generally comprise contacting the drug with a T lymphocyte that expresses a chimeric protein comprising a CD4 molecule lacking the CD4 cytoplasmic domain linked to a src protein tyrosine kinase; stimulating the T lymphocyte; observing the degree of stimulation of the T lymphocyte; and determining whether the drug is capable of blocking T lymphocyte activation therefrom.

The CD4 chimeric proteins of the present invention may also find use in methods for identifying prospective autoantigens in patients with autoimmune diseases. The methods generally comprise transfecting T lymphocytes of the patient with a DNA molecule comprising a nucleic acid sequence encoding a chimeric protein comprising a CD4 molecule lacking the CD4 cytoplasmic domain linked to an src protein tyrosine kinase; contacting the transformed T lymphocytes with antigenic material of the patient; observing stimulation of the T lymphocytes by the antigen; and determining therefrom whether the antigen elicits an immune response in the patient.

Also provided are CD8 chimeric proteins comprising a CD8 extracellular domain, a CD8 transmembrane domain, and an src protein tyrosine kinase. These CD8 chimeric proteins may be employed for identifying MHC Class I restricted antigens. The CD4 and CD8 chimeric proteins of the present invention may also be used for gene therapy to enhance the immune response.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of removal of the kinase domain of a chimeric protein of the present invention to render SH2 more accessible.

FIG. 2A illustrates the general structure of the chimeric proteins of the present invention.

FIG. 2B illustrates T cell surface expression of CD4 and the chimeric proteins of the present invention.

FIGS. 3A and 3B illustrate the antigen-specific response of T cells expressing the CtmLck chimeric protein.

FIGS. 4A and 4B illustrate analysis of T cells expressing chimeric proteins of the present invention that contain non-Lck src family tyrosine kinases.

FIGS. 5A–5C illustrate the effect of ablation of the kinase activity on the function of CtmLck chimeric proteins.

FIGS. 6A and 6B illustrate the role of CD4/Lck chimeric proteins in antibody-mediated T cell stimulation.

FIGS. 7A–7C illustrate the effect of the SH2 domain on the function of chimeric proteins of the present invention.

DESCRIPTION OF SPECIFIC EMBODIMENTS

The present invention provides chimeric proteins having a CD4 extracellular domain, a CD4 transmembrane domain, and an src protein tyrosine kinase. These CD4 chimeric proteins may amplify the response of T cells expressing the proteins in response to immunogenic stimuli, such as antigenic stimulation and antibody cross-linking. These proteins may greatly increase the sensitivity of the immune response (e.g., some CD4 chimeric proteins of the present invention may amplify T cell response by 30 times or more). By amplifying intracellular signals from immunological activation of T cells, the proteins provide a means for identifying drugs that block such intracellular signals. This provides a convenient means for screening potential drugs for the ability to block T cell activation. By blocking T cell activation, the drugs may also block harmful immune responses to antigenic stimuli, such as autoimmune diseases or graft-versus-host disease. Chimeric proteins comprising a CD8 extracellular domain, a CD8 transmembrane domain, and an src protein tyrosine kinase are also provided. These CD8 chimeric proteins may also enhance the immune response and be used to identify drugs that block CD8-mediated immune responses.

Because the chimeric proteins of the present invention may amplify the response of T cells to antigenic stimuli, low level self-antigens (as implicated in the etiology of autoimmune diseases) may be detected and identified by the present invention. DNA encoding the proteins of the present invention may be transfected into T cells of a patient suspected of suffering from an autoimmune disorder. The T cells are then exposed to tissue from the patient and observed for activation. As the T cells express chimeric proteins that amplify activating signals, even small numbers of T cells that react to the self-antigen may be detected. Both the CD4 and CD8 chimeric proteins may be used for gene therapy as described below.

The present invention provides chimeric proteins comprising a CD4 molecule lacking the CD4 cytoplasmic domain linked to an src protein tyrosine kinase. By "chimeric protein" it is meant a protein or peptide that contains subsequences that are substantially homologous to subsequences of at least two different proteins or peptides. By "substantially homologous" it is meant sequences which have at least about 65% relatedness, preferably at least 75%

homology, and more preferably at least about 85–90% or more homology to the amino acid sequence of a naturally occurring CD4 molecule or protein tyrosine kinase of the src family as described in Cooper, *The src Family of Tyrosine Kinases, in Peptides and Protein Phosphorylation, Kemp* and Alewood eds., CRC Press, 1989. These tyrosine kinases include, e.g., $p56^{lck}$ (Lck), c-SRC, Fyn-T, Blk, Yes, Lyn, Fgr, and Hck. The tyrosine kinases may also include fusion proteins constructed from segments of different tyrosine kinases. For example, a fusion between the SH3, SH2, and kinase domains of src and the unique domain of lck is particularly effective for amplifying CD4 mediated T cell signals.

Other tyrosine kinases, such as cytoplasmic tyrosine kinases (e.g., ZAP-70), may also be employed in the chimeric proteins of the present invention. Other non-src family tyrosine kinases may be linked to the extracellular and transmembrane portions of CD4 (or CD8) molecules to form active chimeric proteins that may enhance T cell activation following immunological stimulation.

Thus, it should be understood that the polypeptide compositions of the present invention need not be identical to any particular src protein tyrosine kinase, or amino acid sequence thereof. Unless otherwise indicated, the term "src protein tyrosine kinase" or "src tyrosine kinase" will include homologs, fusions, and fragments of naturally occurring src protein tyrosine kinases as well as other tyrosine kinases, both membrane bound and cytoplasmic.

For example, naturally occurring src tyrosine kinases may be modified by introducing conservative or nonconservative substitutions in the polypeptides, usually fewer than 20 percent, more usually fewer than 10 percent of the amino acids being exchanged. It may be desirable to vary one or more particular amino acids to alter the functional characteristics of the src tyrosine kinase in T cell activation.

Therefore, the present chimeric proteins may be subject to various changes, such as insertions, deletions and substitutions, either conservative or nonconservative, where such changes might provide for certain advantages in their use. "Conservative substitutions" is intended to include, for example, combinations such as gly, ala; val, ile, leu; asp, glu; asn, gln; ser, thr; lys, arg; and phe, tyr. Usually, the sequence will not differ by more than 20% from the sequence of an src tyrosine kinase or amino acid subsequence thereof.

In addition, the amino acid sequence may differ from the natural sequence in the modification of the terminal NH_2 by acylation, e.g., acetylation, or thioglycolic acid amidation, terminal carboxy amidation, e.g., ammonia, methylamine, etc. In some instances, these modifications may provide increased metabolic stability, or the like, as desired.

A particularly useful src tyrosine kinase for use in the present invention is Lck. The Lck tyrosine kinase may be modified as described above for inclusion in the chimeric proteins of the present invention. The function of Lck, when bound to CD4, is not substantially affected by a point mutation that inactivates its phosphotransferase activity. Removal of the catalytic domain may give rise to a co-receptor molecule that functions better than its wild type counterpart. One of Lck kinase roles, observed in the CD4-negative T cell hybridoma transfected with activated Lck and in the Lck-negative mutant Jurkat cells apparently involves the direct or indirect association of Lck with the TCR complex through a CD4 (and CD8)-independent mechanism. In this setting, the kinase function of Lck is most likely required for signal transduction, and enhanced kinase activity may correlate with a lower threshold for activation or with constitutive activation, as exhibited by v-src-transfected hybridoma cells.

A point mutation ablating phosphotransferase function may impair co-receptor activity of the CD4/tyrosine kinase chimera, while truncation of the entire kinase domain may provide a molecule with enhanced activity. Generally, the truncated molecule will lack the putative regulatory tyrosine residues (e.g., tyr-394 and tyr-505 of Lck). Substitution of phe for tyr-505 activates Lck, and there is evidence that a transmembrane protein tyrosine phosphatase, CD45, regulates Lck activity by dephosphosphorylating this residue. In the absence of CD45, this site remains hyperphosphorylated and is thought to interact with the Lck SH2 domain, in either an inter- or intramolecular interaction. As a result, the kinase domain may be sequestered and unable to phosphorylate its appropriate substrates. The phenotypes observed with the kinase-defective chimeric molecules are consistent with this hypothesis, but, in addition, reveal an independent effector function of the SH2 domain of Lck. Reciprocal inhibition of kinase and SH2 activities due to interaction of these domains within Lck may occur in these molecules. Elimination of the kinase domain may render SH2 constitutively accessible, enhancing coreceptor activity despite the loss of phosphotransferase activity (FIG. 1). In the presence of an intact, but inactive, kinase domain decreased activity may occur, which is sensitive to a second mutation that may abolish phosphotyrosine binding by the SH2 domain. Loss of both SH2 and kinase function generally will not, however, completely eliminate coreceptor function (FIG. 6C). This kinase-independent function of CD4-associated Lck may resemble that of proteins that contain only SH2 and SH3 domains, such as Crk and sem-5/GRB2.

In the CD4 chimeric proteins, the src tyrosine kinases are fused to the extracellular and transmembrane domains of CD4 as illustrated in FIG. 2A. Generally, the cytoplasmic domain of CD4 will not be present in the chimeric proteins. The src tyrosine kinase portion of the chimeric protein may be full length or a fragment thereof as described above. The CD8 chimeric proteins of the present invention will be similarly constructed. An src tyrosine kinase will be fused to the extracellular and transmembrane portions of a CD8 molecule.

The present invention also provide DNA molecules encoding the CD4 or CD8 chimeric proteins of the present invention. Generally, the DNA molecules of the present invention will be constructed by synthesis of a gene encoding the fusion protein in a cloning vector. For example, a plasmid encoding CD4 (such as pSM described in Turner et al., *Cell*, 60:755–765 (1990) or pMV7 as described in Glaichenhaus et al., *Cell*, 64:511–520 (1991), both of which are incorporated herein by reference) is subjected to restriction enzyme digestion to introduce a break in the CD4 coding region between the transmembrane and cytoplasmic coding domains. The resulting plasmid may then serve as a source of the CD4 extracellular and transmembrane domains for constructing the hybrids. Plasmids encoding the extracellular and transmembrane portions of CD8α or CD8β may be similarly obtained as described in Littman et al., *Cell*, 40:237–346 (1985) and Normant and Littman, *EMBO J.*, 7:3433–3439 (1988), both incorporated herein by reference.

A DNA sequence encoding an src tyrosine kinase may then be ligated to the transmembrane coding region of the above described plasmid at the restriction site. The resulting plasmid may be used to clone the fusion protein containing the extracellular and transmembrane domains of CD4 and the src tyrosine kinase.

Hybrid DNA technology will generally be employed for expression of the CD4 or CD8 chimeric proteins in trans-

formed T lymphocytes. See, e.g., Sambrook et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989, incorporated herein by reference. For expression and isolation of the chimeric proteins, transfection of non-T lymphocyte host cells may be appropriate, e.g., yeast or procaryotic cells. Techniques for such recombinant production and purification of foreign proteins are well known in the art and briefly described below.

The present invention also provides DNA molecules encoding the chimeric proteins of the present invention. The DNA molecules generally comprise a transcriptional promoter, a DNA sequence encoding the chimeric protein, and a transcriptional terminator.

In general, plasmid vectors containing replication and control sequences which are compatible with the recombinant host cells are used as cloning vectors for the DNA molecules of the present invention. Other vectors, such as λ-phage, cosmids, or yeast artificial chromosomes may also be employed in some instances. The vector ordinarily carries a replication site, as well as sequences which encode proteins that are capable of providing phenotypic selection in transformed cells. For example, *E. coli* may be transformed using pBR322, a plasmid derived from an *E. coli* species. Plasmid pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying and selecting transformed cells. The pBR322 plasmid, or microbial plasmid must also contain, or be modified to contain, promoters which can be used by the microbial organism for an expression of its own proteins. Those promoters most commonly used in recombinant DNA construction include β-lactamase (penicillinase) and lactose promoter systems and a tryptophan (trp) promoter system. One suitable promoter is contained in the in vitro transcription vector pGEM-1. The promoter is a T7 and SP6 polymerase promoter. While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors. The promoters are operably linked to a nucleic acid sequence encoding the chimeric protein. The promoters may be inducible or constitutive and provide a means to express the encoded chimeric protein in the procaryotic host. Following expression, the polypeptide may be purified by standard methods such as described below.

Alternatively, a DNA sequence encoding the chimeric proteins of the present invention may be inserted into a suitable eukaryotic expression vector, which in turn is used to transfect eukaryotic cells. A eukaryotic expression vector, as used herein, is meant to indicate a DNA construct containing elements which direct the transcription and translation of DNA sequences encoding chimeric proteins of interest. Such elements include promoters, enhancers, transcription terminators and polyadenylation signals. By virtue of the inclusion of these elements operably linked within the DNA constructs, the resulting eukaryotic expression vectors contain the information necessary for expression of the polypeptides of interest.

Host cells for use in expressing recombinant chimeric proteins of interest include mammalian, avian, insect and fungal cells. Fungal cells, including species of yeast (e.g., Saccharomyces spp., Schizosaccharomyces spp.) or filamentous fungi (e.g., Aspergillus spp., Neurospora spp.) may be used as host cells for producing chimeric proteins of the present invention. Suitable vectors will generally include a selectable marker, which may be one of any number of genes that exhibit a dominant phenotype for which a phenotypic assay exists to enable transformants to be selected. Preferred selectable markers are those that complement host cell auxotrophy, provide antibiotic resistance or enable a cell to utilize specific carbon sources. The expression units may also include a transcriptional terminator. Choice of a particular host and selectable marker is well within the level of ordinary skill in the art.

Cultured mammalian cells may be used as host cells within the present invention. Cultured mammalian cells for use in the present invention may include human monocytoid, lymphocytoid, and fibroblastoid cell lines. A useful mammalian cell line is the HeLa-tat cells that are HeLa derived cells. Mammalian expression vectors for use in carrying out the present invention will include a promoter capable of directing the transcription of a cloned gene or cDNA. Preferred promoters include viral promoters and cellular promoters. Viral promoters include the immediate early cytomegalovirus promoter (Boshart et al., *Cell* 41:521–530, 1985) and the SV40 promoter (Subramani et al., *Mol. Cell. Biol.* 1:854–864, 1981). Cellular promoters include the mouse metallothionein-1 promoter (Palmiter et al., U.S. Pat. No. 4,579,821), a mouse V_K promoter (Bergman et al., *Proc. Natl. Acad. Sci. USA* 81:7041–7045, 1983); Grant et al., *Nuc. Acids Res.* 15:5496, 1987) and a mouse V_H promoter (Loh et al., *Cell* 33:85–93, 1983).

Such expression vectors may also contain a set of RNA splice sites located downstream from the promoter and upstream from the DNA sequence encoding the polypeptide or protein of interest. Preferred RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the coding sequence of interest.

Polyadenylation signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, ibid.), the polyadenylation signal from the Adenovirus 5 E18region and the human growth hormone gene terminator (DeNoto et al., *Nuc. Acids Res.* 9:3719–3730, 1981). The expression vectors may include a noncoding viral leader sequence, such as the AdenoVirus 2 tripartite leader, located between the promoter and the RNA splice sites. Vectors may also include enhancer sequences, such as the SV40 enhancer and the mouse μ enhancer (Gillies, *Cell* 33: 717–728, 1983). Expression vectors may also include sequences encoding the adenovirus VA RNAs.

Cloned DNA sequences may be introduced into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler et al., *Cell* 14:725, 1978; corsaro and Pearson, *Somatic Cell Genetics* 7:603, 1981; Graham and Van der Eb, *Virology* 52:456, 1973). Other techniques for introducing cloned DNA sequences into mammalian cells, such as electroporation (Neumann et al., *EMBO J.* 1:841–845, 1982), may also be used. In order to identify cells that have integrated the cloned DNA, a selectable marker is generally introduced into the cells along with the gene or cDNA of interest. Preferred selectable markers for use in cultured mammalian cells include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker such as the DHFR gene. Selectable markers are reviewed by Thilly (*Mammalian Cell Technology*, Butterworth Publishers, Stoneham, Mass., which is incorporated herein by reference). The choice of selectable markers is well within the level of ordinary skill in the art.

Transfected mammalian cells are allowed to grow for a period of time, typically 1–2 days, to begin expressing the

DNA sequence(s) of interest. Drug selection is then applied to select for growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker the drug concentration may be increased in a stepwise manner to select for increased copy number of the cloned sequences, thereby increasing expression levels.

The CD4 or CD8 chimeric proteins of the present invention may be purified by a variety of means, including via affinity chromatography, e.g., on an antibody column using antibodies directed against the extracellular or transmembrane domains of CD4, antibodies directed against the extracellular or transmembrane domains of CD8 , antibodies against the src tyrosine kinase, or using CD4 or CD8 binding substances, respectively. Additional purification may be achieved by conventional chemical purification means, such as liquid chromatography, gradient centrifugation, and gel electrophoresis, among others. Methods of protein purification are known in the art (see generally, Scopes, R., Protein Purification, Springer-Verlag, N.Y. (1982), which is incorporated herein by reference) and may be applied to the purification of the recombinant chimeric proteins described herein; see also a purification protocol described in U.S. Pat. No. 4,929,604, incorporated herein by reference.

The CD4 chimeric proteins of the present invention may replace wild-type CD4 as a co-receptor during T cell activation. Association of CD4 extracellular and transmembrane domains with an src tyrosine kinase provides a means to mediate the function of the CD4 cytoplasmic domain in this system. The activity of the chimera required a functional CD4 extracellular domain as the chimeric protein are generally sensitive to mutations in the putative MHC class II binding site of CD4 and may be completely blocked by antibodies against CD4.

Likewise, the CD8 chimeric proteins of the present invention may replace wild-type CD8 proteins as co-receptors for activation of cytotoxic T cells. The CD8 chimeric proteins may amplify CD8 mediated T cell activation in cytotoxic lymphocytes.

Nucleic acid encoding chimeric proteins as described above may be introduced into T cells for expression. The nucleic acid may be DNA or RNA. The nucleic acid may be introduced into the T cells in by a variety of methods well known in the art and described above. Generally, the nucleic acid encoding the chimeric proteins will be introduced into the T cells by means of a retroviral vector, such as pMV7.

The retroviral vectors may be prepared as described in Glaichenhaus et al., *Cell*, 64:511–520 (1991), incorporated herein by reference. Briefly, nucleic acid encoding the chimeric proteins is introduced into a packaging cell line such as PA317 as described by Miller and Buttimore, *Mol. Cell. Biol.*, 6:2895–2902 (1986), incorporated herein by reference. Following incubation, culture supernatants are harvested and used to infect a Psi-2 cell line that provides for selection of a genetic marker carried by retroviral vectors containing nucleic acid encoding the chimeric protein. Following selection, viral stock is isolated from the infected cell line. The viral stocks may then be used to infect a T lymphocyte cell line, such as 171 cells. For screening potential drugs, it is desirable that the infected cell line be activated by a known antigen.

The infected cells may be screened to identify those cells that express the chimeric proteins. Generally, FACS analysis employing a monoclonal antibody to the CD4 region of the chimeric protein will be used to identify cells expressing the chimeric protein. Generally, analysis is facilitated if the infected T cell line does not express CD4, such as the 171.3 cell line. Alternatively, comparison of the staining pattern of infected and uninfected control T cells may be used to identify cells expressing the chimeric protein. These cells may also be sorted by FACS or magnetic beads and panning.

T cells that express the chimeric proteins of the present invention may be employed in methods for screening drugs that block T cell activation. The methods generally comprise contacting the drug with a T lymphocyte that expresses a chimeric protein comprising a CD4 molecule lacking the CD4 cytoplasmic domain linked to an src protein tyrosine kinase; stimulating the T lymphocyte; observing the degree of stimulation of the T lymphocyte; and determining whether the drug is capable of blocking T lymphocyte activation therefrom.

The T lymphocyte may be stimulated by a variety of means. For example, the T cell may be stimulated by exposure of the T cell to an antigen recognized by the T cell. Generally, exposure of the T cell to the antigen will occur in the presence of antigen-presenting cells to enhance stimulation and activation of the T cell.

Antibody cross-linking of T cell receptors (TCRs) on the cell surface may be employed to stimulate the T cells. While TCR cross-linking will initiate T cell immunological function, CD4 interaction (and associated tyrosine kinase activity) with cross-linked TCRs is required for the T cell to become fully activated. Thus, anti-TCR and anti-CD4 antibodies may be employed for T cell stimulation. Antibody cross-linking of CD4 chimeric proteins of the present invention may also be employed to activate T cells if forms of the src chimeras can activate in the absence of TCR cross-linking (e.g., carboxy-terminal mutations of src kinases, such as a Phe to Tyr substitution at amino acid position 505 in Lck or position 527 in SRC).

The degree of stimulation of the T cells is then observed. Among the many ways the degree of T cell stimulation may be qantified, measuring observable products of T cell activation is often the most convenient. Such products include interleukin-2 synthesis. Supernatant from stimulated T cells may be titrated in dilutions with cytotoxic T lymphocytes. Proliferation of cytotoxic T lymphocytes may then be induced by treatment with a mitogen such as 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide. Drugs blocking T cell activation will generally decrease the synthesis of interleukin-2 by stimulated T cells.

Alternatively, rapid colorimetric assays for interleukin-2 promoter driven reporter molecules may be employed. Such reporter molecules include, e.g., secreted alkaline phosphatase and β-galactosidase. Assays for such reporter molecules are well known in the art and may provide an indirect means for identifying activation of the interleukin-2 promoter, and hence indicate increased interleukin-2 production.

Alternative means of measuring the degree of T cell stimulation include, e.g., determining the degree of activity of the src protein tyrosine kinases in stimulated T cells. As the tyrosine kinases will phosphorylate tyrosine residues following T cell activation, quantification of such phosphorylation may be used to assess the degree of blocking of T cell activation by a drug. Intracellular protein phosphorylation may be determined by immunoblotting with monoclonal antibodies specific for phosphorylated tyrosine residues.

The chimeric proteins of the present invention may also be employed in methods for detection of low level self-antigens suspected of causing autoimmune disease. The

methods generally comprise transfecting T lymphocytes of the patient with a DNA molecule comprising a nucleic acid sequence encoding a chimeric protein comprising a CD4 molecule lacking the CD4 cytoplasmic domain linked to an src protein tyrosine kinase; contacting the transformed T lymphocytes with the patient's antigen; observing stimulation of the T lymphocytes by the antigen; and determining therefrom whether the antigen elicits an immune response in the patient. Cells expressing CD8 chimeric proteins may be employed in similar methods for identifying low-level MHC Class I restricted antigens.

The patient's T cells may be transfected with DNA encoding a chimeric protein of the present invention by a variety of means well known to those of skill in the art. Generally, a retroviral vector as described above will be employed to transfect the patient's T cells. Alternatively, other methods for transfecting eukaryotic cells, such as calcium phosphate-mediated transfection or electroporation. Typically, chimeric proteins having more activity than naturally occurring src protein kinases will be employed in these screening methods. Cells expressing the chimeric proteins of the present invention may be identified by FACS as described above.

Following identification of cells expressing the chimeric proteins of the present invention, the cells are contacted with the suspected antigen. To demonstrate that patient T cells react with a self-antigen, a crude extract from patient tissue may be employed to stimulate the T cells. As the transfected patient T cells will generally exhibit a greater degree of activation to antigenic stimulation, even self-antigens present in low levels may be detected by observing the transfected T cells. Observation of T cell activation, e.g., by increased interleukin-2 synthesis or increased protein tyrosine phosphorylation, may indicate that T cells of the patient may be activated by a self-antigen.

Self-antigens may also be identified by similar methods. Fractions of crude patient tissue extracts may be tested against patient T cells transfected with DNA encoding chimeric proteins of the present invention. Identification of T cell activation in a fraction indicates that a self-antigen is present in that fraction. The active fraction may then be fractionated and the resulting samples similarly tested against the patient's transfected T cells. This process may be repeated until the antigen is purified. The purified antigen may then be identified by methods well known to those of skill in the art.

DNA molecules encoding the chimeric proteins of the present invention may be employed for gene therapy for enhancing a desired immune response. Antigen-specific T cells could be isolated from a patient and transfected with DNA of the present invention. Expression of the encoded chimeric proteins could be determined and the cells reinfused into the patient. The transformed cells expressing the chimeric proteins may demonstrate an enhanced immune response to the chosen antigen, thereby enhancing immunological removal of the antigen.

One particularly useful application of gene therapy employing the DNA molecules of the present invention is enhancement of cytotoxic T cell activity against tumors. Cytotoxic T cells specific for the tumor may be isolated from tumor tissue. The T cells may be transfected with a DNA molecule encoding a CD8 chimeric protein, such as a CD8-Lck chimera. The cells may be re-infused into the patient and exhibit enhanced tumor killing ability. Such treatment is especially attractive as T cells from animals with malignancies have been shown to have a reduced level of tyrosine kinases.

The following examples are offered by way of illustration and not limitation.

EXAMPLES

The following enzymes and antibodies were used in the following examples. The Pfu polymerase was purchased from Strategene. MAbs GK1.5 (anti-murine CD4), 2C11-145 (anti-murine, CD3), 519 (anti-mouse β2-microglobulin), and 4G10 (anti-phosphotyrosine) were used as hybridoma culture supernatants. The phycoerythrin-conjugated GK1.5 was purchased from Becton Dickinson. Peroxidase-labeled goat anti-rabbit IgG was purchased from Kirkegaard & Perry Laboratories. Goat anti-mouse IgG-POD conjugate was from Boehringer Mannheim.

Cell lines used in the following examples were as follows. The T cell hybridoma 171 has been previously described (Glaichenhaus et al., *Cell*, 64:511–520 (1991), incorporated herein by reference). The cell line produces monoclonal antibodies specific for a hen egg lysozyme peptide corresponding to residues 74–88 (NLANIPASALLSSDI) in association with the I-A^b MHC molecule. A subclone of this cell line, 171.3, was used in all experiments. The FT7.1 cell line is a derivative of L cells that express both the α and β chains of the A^b MHC class II molecule (Ronchese et al., *J. Immunol.*, 139:629–638 (1987), incorporated herein by reference). All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FCS), 50 μM 2-mercaptoethanol, 100 Unit/ml penicillin G, and 100 μg/ml streptomycin. For the IL-2 indicator cell line CTLL, the medium also contained 10 mM Hepes, pH7.4, vitamins and nonessential amino acids in addition to 60 unit/ml recombinant human IL-2 (purchased from Amgen or R&D; the activity of the R&D product was normalized to that of the Amgen product).

Transient transfection of COS7 cells with CD4-based constructs and immunoprecipitation analyses and kinase assays of the chimeric proteins were performed as previously described (Turner et al., *Cell*, 60:755–765 (1990), incorporated herein by reference. T cells were analyzed by the following procedure: 10^7 cells were pelleted and washed once with cold PBS. The cells were lysed in 1 ml lysis buffer (137 mM NaCl , 1 mM EDTA, 10% glycerol, 0.5% Triton X-100, and 50 mM Tris-HCl, pH 8.0) in the presence of protease inhibitors (10 μg/ml leupeptin, 10 μg/ml soy bean trypsin inhibitor, 2 μg/ml pepstatin, 500 unit/ml aprotinin, and 1 mM PMSF) and the tyrosine phosphatase inhibitor sodium orthovanadate at 2.5 mM. The lysate was cleared by a 5 minute centrifugation at 4° C. and then incubated with 40 μl of goat anti-rat IgG-Sepharose beads (Sigma) pro-armed with the anti-CD4 MAb GK1.5. Immunoprecipitation was allowed to continue for 2 hours at 4° C. The beads were washed 3 times with cold PBS, 5 mM EDTA, 0.5% NP-40. The beads were then resuspended in 1 ml kinase buffer (10 mM $MnCl_2$ and 50 mM Tris-HCl, pH7.5) and divided into 30% for the kinase assay and 70% for immunoblotting. For the kinase assay, the beads were incubated in 15 μl kinase buffer containing 20 μCi λ^{32}P-ATP (3000Ci/mM, Amersham) and 10 μg acid-denatured enolase (Sigma). After 5 minutes at room temperature, 15 μl of 2 X nonreducing SDS sample buffer was added to stop the reaction. The reaction product was analyzed by SDS-PAGE. The gel was fixed, treated with 1M KOH at 55° C. for 1 hour to remove serine/threonine phosphorylation, and refixed before being dried down for autoradiography. For immunoblotting, the beads were denatured in nonreducing sample buffer. Half of each sample was electrophoresed on SDS-PAGE and trans-

ferred to a nitrocellulose membrane. The filter was blocked in 5% dried milk in TBST (10 mM Tris. Cl, pH8, 0.9% NaCl, and 0.1% Tween-200, incubated in appropriately diluted rabbit antiserum against murine CD4 or Lck, followed by peroxidase-labeled anti-rabbit IgG (1:10,000 dilution), and then developed using the ECL detection system (Amersham).

Antibody-mediated crosslinking was performed as follows: 10^7 T cells were centrifuged and washed once with cold media without FCS. The washed cells were incubated on ice with 0.5 ml of the appropriate MAb(s) at saturating concentration for 30 minutes. Fourteen mls of cold PBS was added to the tube and the cells were collected by centrifugation. The cells were resuspended in 100 µl media without FCS and transferred to an eppendorf tube. After 3 minutes at 37° C., 2 µg of rabbit, anti-hamster IgG was added to the cells. The tube was immediately returned to 37° C. and the incubation continued (usually for 2 minutes, except for the time course experiments). The cells were lysed on ice by mixing quickly with 100 µl of ice-cold 2 x lysis buffer which contained 2% NP-40 (instead of Triton X-100) and inhibitors of proteases and phosphatases. The lysate was cleared by centrifugation. For blotting of total cellular proteins, the lysate was denatured by the addition of 100 µl 2 x nonreducing SDS sample buffer. For immunoprecipitation of Lck 800 µl of cold lysis buffer without detergent was added, and the lysate was incubated with MAb A4 (anti-human Lck)-armed anti-mouse agarose beads for two hours at 4° C. The beads were washed 3 times with TBST+10 mM EDTA+1 mM sodium orthovanadate before addition of 40 µL of 2 x nonreducing sample buffer. Denatured samples were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Proteins phosphorylated on tyrosine residues were detected by immunoblotting using MAb 4G10 as the primary antibody followed by peroxidase coupled goat anti-mouse IgG at 1:20,000 dilution. The blot was developed by the ECL method.

EXAMPLE 1

Construction of Plasmids Encoding the Chimeric Proteins

This example demonstrates construction of plasmids encoding chimeric proteins of the present invention. Several of the chimeric proteins contained subsequences having mutations of the corresponding wild-type protein. The plasmids produced in this example provided a source of chimeric proteins for use in assays.

A Xba I site was introduced into the mouse CD4 cDNA in the pSM vector (Turner et al., *Cell*, 60:755–765 (1990), incorporated herein by reference) at residue 394 (the end of the transmembrane domain) by oligonucleotide-directed mutagenesis, resulting in a serine substitution for cys-395 (the numbering of the residues is according to Littman and Gettner, *Nature*, 352:453–455 (1987)). The resulting plasmid was used as the source of the CD4 extracellular and transmembrane domains for constructing the hybrids. A Xho I site was introduced into tho murine lck cDNA in pSM at the sequence corresponding to amino acid residue 5. (Marth et al., *Cell*, 43:393–404 (1985).

The CD4 and Lck sequences were ligated together in the presence of a pair of annealed complementary oligonucleotides bridging the 3' CD4 Xba I and the 5' lck Xho I sites, giving rise to CtmLck containing the CD4 cDNA up to residue 396 and the whole Lck sequence (residues 1–509 with the first amino acid replaced by lysine). To construct other chimeras, the entire coding sequences of chicken c-src (Takeya and Hanafusa, *Cell*, 32:881–890 (1983), mouse fyn-T (Cooke and Perimutter, *Few Biologist*, 1:66–74 (1990) and human hck (Quintrell et al., *Mol. Cell. Biol.*, 7:2267–2275 (1987) were amplified by the polymerase chain reaction using the Pfu polymerase, which has a low mutation frequency, under conditions suggested by the manufacturer. The 5' primer contained a Xba I site to facilitate the ligation of the PCR products with the CD4 cDNA missing the cytoplasmic domain. The junctions between CD4 and src family kinases were sequenced.

FIG. 2A is a schematic representation of the chimeras between CD4 and src-family PTK's. The extracellular, transmembrane, and cytoplasmic domains of CD4 and the unique N-terminal, SH3, SH2, and kinase domains of the PTK's are indicated. The drawing is not to scale. The chimeras are designated by Ctm followed by the name of the particular kinase. For example, CtmLck indicates the hybrid of CD4 and Lck.

Chimeras containing various mutations were generated by a 2-step process: the mutations were first introduced into lck by oligonucleotide-directed mutagenesis; then part of the lck cDNA containing the mutation was swapped into the chimera using standard methods. In the CtmLck273R mutant, lys-273 of Lck, which is essential for kinase activity, was substituted with arginine; in the CtmLck154K mutant, arg-154 of Lck, which is predicted to be critical for phosphotyrosine-binding, was replaced with lysine; the CtmLck273R154K mutant was obtained by combining both of the above mutations; in the kinase domain truncation mutant, residues 249–505 of lck were deleted using a bridging oligonucleotide. Mutations at the putative CD4 MHC class II binding site (the MM4 mutants) were generated by changing residues 101–107 (Lys-Val-Thr-Phe-Ser-Pro-Gly) to Gly-Leu-Thr-Thr-Thr-Thr-Thr. All the mutations were verified by sequencing.

The chimeras were initially made in the pSM vector for analysis in COS7 cells. They were subsequently cloned into the pMV7 vector for preparation of retroviral packaging cell lines (Glaichenhaus et al., supra).

EXAMPLE 2

Preparation of Retroviral Packaging Cell Lines and Isolation of Infected T Cells

This example demonstrates preparation of packaging cells producing retroviral vectors containing chimeric proteins of the present invention. Following preparation of the packaging cells, T lymphocytes were infected with retroviral vectors encoding chimeric proteins.

The preparation of the packaging cells producing retrovirus encoding the various CD4-containing proteins and the subsequent infection of 171.3 cells were as described by Glaichenhaus et al., supra. Cells expressing CD4 on their surface were either selected by FACS (Glaichenhaus et al., supra) or sorted using magnetic beads, as outlined below. Two days after infection, 10^7 T cells were incubated with the anti-CD4 MAb GK1.5 at saturating concentration for 30 minutes on ice. The cells were washed extensively with phosphate-buffered saline (PBS) plus 2% FCS. The cells were mixed with 4×10^8 Dynabeads M-450 coated with sheep anti-rat IgG (Bioproducts for Sciences, Inc.), pelleted, and allowed to stand on ice for 30 minutes. The cell/bead mixture was resuspended in 100 µl PBS plus 2% FCS and the incubation was continued for another 30 minutes. Cells

bound to beads were separated from the unbound cells by magnetic force. The beads and cells were resuspended in 5 ml PBS plus 2% FCS and then isolated again using the magnet. This process was repeated an additional 3 times. After three days in culture, bead-purified cells that had detached from the beads were separated and discarded, and the culture was continued for three more days. The remaining cells that had detached from the beads were expanded. To obtain cells expressing similar levels of surface CD4, the above sorted cell lines were resorted by FACS, using a narrow window of fluorescence intensity.

EXAMPLE 3

Analysis of Cell Surface CD4 Expression

This example demonstrates detection of cell surface expression of CD4 antigens on transfected cells.

Approximately 5×10^5 cells (either transfected COS7 cells or sorted T cells) were stained with 100 μl phycoerythrin-conjugated GK1.5 at saturating concentration (1:200 dilution) and then resuspended in PBS, 2% FCS, and 0.2 μg/ml propidium iodide. Cell surface fluorescence was analyzed on a FACScan (Becton-Dickinson), and the dead cells were gated out by their high propidium iodide staining.

The 171.3-derived cells were stained with phycoerythrin-conjugated anti-CD4 MAb GK1–5 and analyzed by flow cytometry. The parental 171.3 cells do not express CD4 and represent the negative staining profile (unfilled line in each panel). Each cell population was obtained by cell sorting following infection with retrovirus encoding the following: wild type CD4 (CD4); CD4 with a cysteine to alanine substitution in the cytoplasmic domain (CD4MCA1); CD4 mutated in the MHC class II binding site (CD4MM4); CD4/lck chimera (CtmLck); CD4/lck chimeras containing mutations in the class II binding site (CtmLckMM4), the catalytic domain (CtmLck273R), the SH2 domain (CtmLck154K), and both the kinase and SH2 domains (CtmLck273R154K); CD4/lck chimera lacking the kinase domain (CtmLckakin); CD4 chimeras containing Src, Fyn T, and Hck (CtmSrc,CtmFynT, and CtmHck).

The different sorted cell lines expressed roughly equivalent levels of both surface CD4 (FIG. 2B) and TCR/CD3. FACS analyses showed that all of the chimeric molecules were expressed at similar levels on the cell surface (FIG. 2B).

EXAMPLE 4

Function of CD4/Lck Chimeric Protein During T cell Activation

This example demonstrates that CD4/LCK chimeric molecules may function as a co-receptor during T cell activation. T cells expressing the CD4/Lck chimeric protein were readily activated upon stimulation with the T cell-specific antigen.

A DNA sequence encoding a chimeric protein consisting of the extracellular and transmembrane domains of CD4 linked to the entire Lck protein was constructed as described in Example 1. The CD4 cytoplasmic domain was omitted from this hybrid protein to avoid CD4 interaction with endogenous T cell Lck. The cDNA was first cloned into an SV40-based vector (pSM) and shown to direct expression of surface CD4 upon transient transfection into COS7 cells. The fusion protein was immunoprecipitated from lysates of these cells with anti-CD4 antibody and demonstrated to have in vitro kinase activity. The construct was then introduced via retrovirus-mediated gene transfer into the antigen-specific T cell hybridoma line 171.3 that requires CD4 expression for activation (Glaichenhaus et al., supra).

Activation of the 171 cells and measurement of IL-2 production were as described by Glaichenhaus et al., supra, previously incorporated herein by reference. For the antibody blocking experiments, 25 μl of the anti-CD4 or anti-β2-microglobulin hybridoma supernatants was also added to the mixture of T cells, antigen, and antigen presenting cells.

Infected cells were sorted to establish a polyclonal cell line expressing the chimeric protein. The same approach was used to prepare cell lines expressing the other constructs described below. The CD4+ cell lines were then tested for their response to stimulation with the antigen, a synthetic peptide analog of hen egg lysozyme, in the presence of antigen-presenting cells expressing the appropriate class II molecule, I-A^b. FIG. 3A illustrates a comparison of responses of T cells expressing no CD4 (the parental 171.3 cells), the CD4/lck chimeric molecule (CtmLck), wild type CD4, mutant CD4 (MCA1) that does not bind $p56^{lck}$, and mutant CD4 (MM4) or CD4/lck chimera (CtmLckMM4) that do not bind MHC class II. The T cells were stimulated by co-culturing with the appropriate antigen presenting cells and the peptide antigen at indicated concentrations.

In contrast to the parental CD4-negative 171.3 cells, these cells (CtmLck) were readily activated upon antigen stimulation, as were cells that expressed wild type CD4 (FIG. 3A). As was previously demonstrated, a form of CD4 with a mutation in one of the cysteine residues required for binding of endogenous Lck (CD4MCA1) mediated a barely-detectable response to antigen. Thus, the chimeric molecule functionally resembles wild-type CD4, to which Lck is bound non-covalently.

Activated forms of src family kinases can increase IL-2 responses in the absence of a co-receptor interaction with ligand. For example, overexpression of an activated form of Lck has been previously shown to enhance responsiveness of a CD4- T cell hybridoma to stimulation with antigen (Abraham et al., *Nature*, 350:62–66 (1991). In addition, expression of viral src in another T cell line resulted in constitutive IL-2 expression (O'Shea et al., 1991). In order to rule out the possibility that the mode of action of the CD4/lck chimera was through constitutive activity of the PTK that lowered the threshold for activation, the reported MHC class II binding site of CD4 (Lamarre et al., *Science*, 245:743–746 (1989); Clayton et al., *Nature*, 339:548–551 (1989), both of which are incorporated herein by reference) was mutated and assayed for functional activity. This mutation completely abolished the function of both CD4 and the CD4/lck chimera (CD4MM4 and CtmLckMM4 in FIG. 3A). The defect in these molecules was confined to the function of the extracellular domain, since antibody-mediated crosslinking of CD3 and the mutant CD4 or CD4/lck chimera resulted in normal levels of tyrosine phosphorylation of cellular substrates and the in vitro kinase activity of the MHC-non-binding mutant chimeric molecule was similar to that of the CtmLck molecule. In addition, the activity of the chimeric molecule in the T cell hybridoma was completely blocked by antibody against CD4 but not by a control MAb against β_2-microglobulin used as a control (FIG. 3B). These results indicate that, like wild type CD4, a functional class II-binding extracellular domain is essential for the activity of the CD4/lck hybrid molecule and confirms that this molecule is appropriately regulated in the 171.3 hybridoma.

EXAMPLE 5

Function of CD4 Chimeric Proteins Having a non-Lck Tyrosine Kinase

This example describes functional assays of CD4 chimeric proteins having non-Lck src tyrosine kinases. The chimeric proteins displayed co-receptor function in response to antigen.

Polyclonal populations of cells derived from the 171.3 hybridoma expressing the various chimeric proteins were prepared and tested for their response to antigen. The expression of the T cell receptor complex was nearly identical in all cells with the exception of those expressing CtmHck, whose TCR/CD3 level was approximately 60% that of the other lines. Chimeric proteins were precipitated from the sorted cell lines using anti-CD4 antibodies. Acid-denatured enolase was included in the kinase reactions to serve as a model substrate. The reaction was analyzed on 10% SDS-PAGE. The chimeric proteins were shown to have kinase activity (FIG. 4A).

All chimeric proteins displayed co-receptor function in response to antigen when co-cultured with antigen-presenting cells and peptide antigen (FIG. 4B), although cells expressing the CD4/hck hybrid protein were considerably less responsive than cells expressing the other molecules. The results indicate that, when appropriately localized within the cell, e.g. tethered to CD4, different members of the src family may be functionally interchangeable.

EXAMPLE 6

An Active Kinase Domain is not Essential for the Function of the CD4/lck Chimera

This example demonstrates that the tyrosine kinase domain of the chimeric proteins need not be active to provide a functional chimeric protein.

Protein phosphorylation on tyrosine residues has been shown to play an essential role in TCR-mediated activation of T cells (June et al., *Proc. Natl. Acad. Sci. USA*, 87:7722–7726 (1990). In addition, crosslinking of CD4 to the TCR/CD3 complex results in a significant enhancement in cytoplasmic PTK activity and in signal transduction (June et al., supra). Two forms of the CD4/lck chimera that were defective in phosphotransferase activity and expressed these in the T cell hybridoma were prepared. The chimeras were immunoprecipitated using an anti-CD4 MAb from T cell lysates; kinase reactions were initiated by addition of γ-32P-ATP. Acid-denatured enolase was used as a model kinase substrate. The reaction products were separated by SDS-PAGE and visualized by autoradiography.

Both a point mutation in a residue essential for phosphotransferase activity (CtmLck273R, lys-273 of Lck converted arg) or a truncation of the entire, kinase domain (CtmLck-akin, missing residues 249–505 of Lck) resulted in complete loss of the in vitro kinase activity of the chimeric proteins. (FIGS. 5A & 5B). The mutant CD4/lck lacking the entire catalytic domain rendered the hybridoma highly responsive to antigen stimulation when co-cultured with peptide antigen at various concentrations in the presence of antigen-presenting cells (FIG. 5C). The chimera with a substitution for lys-273 also showed substantial, though reduced, co-receptor activity. A CD4/hck chimeric molecule with an analogous point mutation also retained biological activity that was similar to that of its wild-type counterpart. These results indicate that, in antigen-specific T cell activation, the function of CD4-associated Lck does not exclusively rely on its enzymatic activity and that non-catalytic properties of src family PTK's can contribute directly to at least some of their functions.

Previous studies have shown that antibody crosslinking of the TCR-complex leads to the activation of a protein tyrosine kinase pathway, and that co-crosslinking of CD4 with the TCR significantly enhances this process (June et al., *J. Immunol.*, 144:1591–1599 (1990)). To determine whether this enhancement is dependent on the catalytic activity of CD4-associated Lck, the effects of the wild type and the kinase-defective CD4/lck proteins on TCR-induced protein tyrosine phosphorylation were analyzed using antiphosphotyrosine immunoblots. Crosslinking was performed with the indicated MAbs against murine CD4 or/and CD3 plus secondary antibodies at 37° C. for 2 minutes. Phosphotyrosine-containing proteins were detected by immunoblotting. Lysate from 1.5×10^6 cells was applied to each lane. T cells expressing the CD4/lck chimera are in lanes 1–4 and its kinase-negative mutant lanes 5–8 in FIG. 6A. Aggregation of the CD4/lck chimera (approximately 108 kd) with anti-CD4 antibody resulted in its own tyrosine phosphorylation (lane 2). Treatment with anti-CD3 antibody resulted in the specific tyrosine phosphorylation of several proteins, with prominent bands observed at 36 kd, 72 kd, and 110 kd. Coaggregation of the chimera with the TCR/CD3 complex significantly enhanced the tyrosine phosphorylation of these cellular proteins (lanes 3 and 4). In contrast, crosslinking of the kinase-defective mutant chimera did not result in its phosphorylation (lane 6), nor was protein tyrosine phosphorylation significantly augmented by its co-crosslinking with the TCR/CD3 complex (lanes 7 and 8). Similar results were also obtained in a time course study.

These data indicate that it is unlikely that the kinase-defective mutant chimeric proteins function in the antigen-specific response due to their association with an endogenous kinase (including Lck). This conclusion is reinforced by the inability to co-immunoprecipitate endogenous Lck or another kinase activity with the chimera under a variety of different conditions. To further examine the possibility of a functional association between endogenous Lck and the chimeric protein, the tyrosine-phosphorylation status of Lck following antibody-mediated crosslinking of the kinase-negative chimera was analyzed (FIG. 6B). Crosslinking was carried out for 2 minutes at 37° C. Lck was then immunoprecipitated from the cell lysates and analyzed by anti-phosphotyrosine immunoblot. Cells expressing wild type CD4 were used as a positive control (lanes 1–2).

Immunoprecipitated Lck from lysates of untreated or anti-CD4 treated cells was analyzed by immunoblotting with an anti-phosphotyrosine MAb. Crosslinking of wild type CD4 resulted in the heavy phosphorylation of Lck on tyrosine, but no such change was detectable, in cells expressing the kinase-deficient chimeric protein. Reprobing of the same niter with anti-Lck antibodies following stripping revealed similar levels of Lck in all immunoprecipitates.

EXAMPLE 7

The SH2 Domain of Lck Mediates the Co-Receptor Activity of the CD4/lck Chimera

This example demonstrates that the SH2 domain of Lck is involved in mediating co-receptor activity of the chimeric proteins.

The role of the Lck SH2 domain, which is predicted to bind to specific tyrosine-phosphorylated cellular proteins was analyzed. A point mutation (arg>lys) was introduced at the highly conserved arg154 residue, which is predicted to be located in the phosphotyrosine-binding cleft based on the crystal structure of the homologous v-src SH2 domain (Waksman et al., *Nature*, 358:646–653 (1992), incorporated herein by reference). A similar change in the abl SH2 domain has been shown to abrogate its binding to tyrosine-phosphorylated proteins (Mayer at al, *Mol. Cell, Biol.*, 12:609–618 (1992), incorporated herein by reference. Although this mutation did not affect the ability of the chimera to phosphorylate substrates in vitro (FIGS. 7A & 7B), the mutant protein showed decreased co-receptor activity (CtmLck154K construct in FIG. 7). Significantly, when this SH2 domain mutation was combined with the point mutation (arg-273) that abolished kinase activity, the resulting defect was much more severe than that observed with either mutation alone (FIG. 7C, compare the CtmLck273R154K double mutant with the CtmLck154K and CtmLck273R mutants). The phenotype of the double mutant indicates that Lck works through multiple activities contributed independently by its various domains.

All publications, patents and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

What is claimed is:

1. A chimeric protein comprising a CD4 molecule lacking the CD4 cytoplasmic domain linked to an src protein tyrosine kinase.

2. A chimeric protein as in claim 1, wherein the CD4 molecule is a human CD4 molecule.

3. A chimeric protein as in claim 1, wherein the src protein tyrosine kinase is a human src protein tyrosine kinase.

4. A chimeric protein as in claim 3, wherein the src protein tyrosine kinase is $p56^{lck}$.

5. A chimeric protein as in claim 3, wherein the src protein kinase is c-SRC, Fyn-T, ZAP-70, or Hck.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,504,000
DATED : April 2, 1996
INVENTOR(S) : LITTMAN et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In column 1 at line 5, insert the following statement:
--This invention was made with Government support under Grant No. AI23513, awarded by the National Institutes of Health. The Government has certain rights in this invention.--

Signed and Sealed this

Twenty-second Day of June, 1999

Attest:

Q. TODD DICKINSON

Attesting Officer

Acting Commissioner of Patents and Trademarks

US005837544A

United States Patent [19]

Capon et al.

[11] Patent Number: 5,837,544

[45] Date of Patent: Nov. 17, 1998

[54] **METHOD OF INDUCING A CELL TO PROLIFERATE USING A CHIMERIC RECEPTOR COMPRISING JANUS KINASE**

[75] Inventors: **Daniel J. Capon**, Hillsborough; **Huan Tian**, Cupertino; **Douglas H. Smith**, Foster City; **Genine A. Winslow**, Hayward, all of Calif.; **Miriam Siekevitz**, New York, N.Y.

[73] Assignee: **Cell Genesys, Inc.**, Foster City, Calif.

[21] Appl. No.: **485,293**

[22] Filed: **Jun. 7, 1995**

Related U.S. Application Data

[63] Continuation of Ser. No. 382,846, Feb. 3, 1995.

[51] **Int. Cl.**[6] **C12N 5/10**; C12N 1/38

[52] **U.S. Cl.** **435/375**; 435/325; 435/69.7; 435/376; 435/377

[58] **Field of Search** 536/23.4; 435/69.7, 435/240.2, 320.1, 325, 375, 376, 377; 530/387.3

[56] **References Cited**

U.S. PATENT DOCUMENTS

5,359,046	10/1994	Capon et al.	536/23.4
5,470,730	11/1995	Greenberg et al.	435/172.3
5,504,000	4/1996	Littman et al.	435/194

FOREIGN PATENT DOCUMENTS

0340793	8/1989	European Pat. Off. .
WO9319163	3/1993	WIPO .
WO9429438	12/1994	WIPO .

OTHER PUBLICATIONS

Nakamura et al. (1994) Nature 369: 330–333.

Miyazaki et al. (1994) Science 266: 1045–1047.

Heinzel et al., *Use of Simian Virus 40 replication to amplify Epstein–Barr virus shuttle vectors in human cells*, J.Virol. 63:3738–3746 (1988).

Kishimoto et al., *Cytokine Signal Transduction*, Cell 76:253–262 (1994).

Morgan and Anderson, *Human gene therapy*, Ann.Rev.Biochem. 62:191–217 (1993).

Mulligan, *The basic science of gene therapy*, Science 360:926–930 (1993).

Nienhuis et al., *Gene Transfer into Hematopoietic Stem Cells*, Cancer 67:2700–2704 (1991).

Orkin et al., Report and Recommendations of the Panel to Assess the NIH Investment in Gene Therapy, (1995).

Paul, *Tumor Immunology*, Fundamental Immunol., Chap. 34, pp. 923–955 (1989).

Riddell et al., *Genetically modified T–cell clones as a treatment for human viral diseases*, (Meeting Soc.Biol.Therapy (Abstract), p. 50 (1993).

Sakai et al., *Signal transduction by a CD16/CD7/Jak2 fusion protein*, J.Biol.Chem. 270:18420–18427, (1995).

Spencer et al., *Controlling signal transduction with synthetic ligands*. Science 262:1019–1024 (1993).

Stancovski et al., *Targeting of T lymphocytes to Neu/HER–2–expressing cells using chimeric single chain Fv receptors*, J.Immunol. 151:6577–6582 (1993).

Travis, *Making molecular matches in the cell*, Science 262:989 (1993).

Weatherall, *Scope and limitations of gene therapy*, British Med.Bull. 51:1–11 (1995).

Primary Examiner—Stephen Walsh

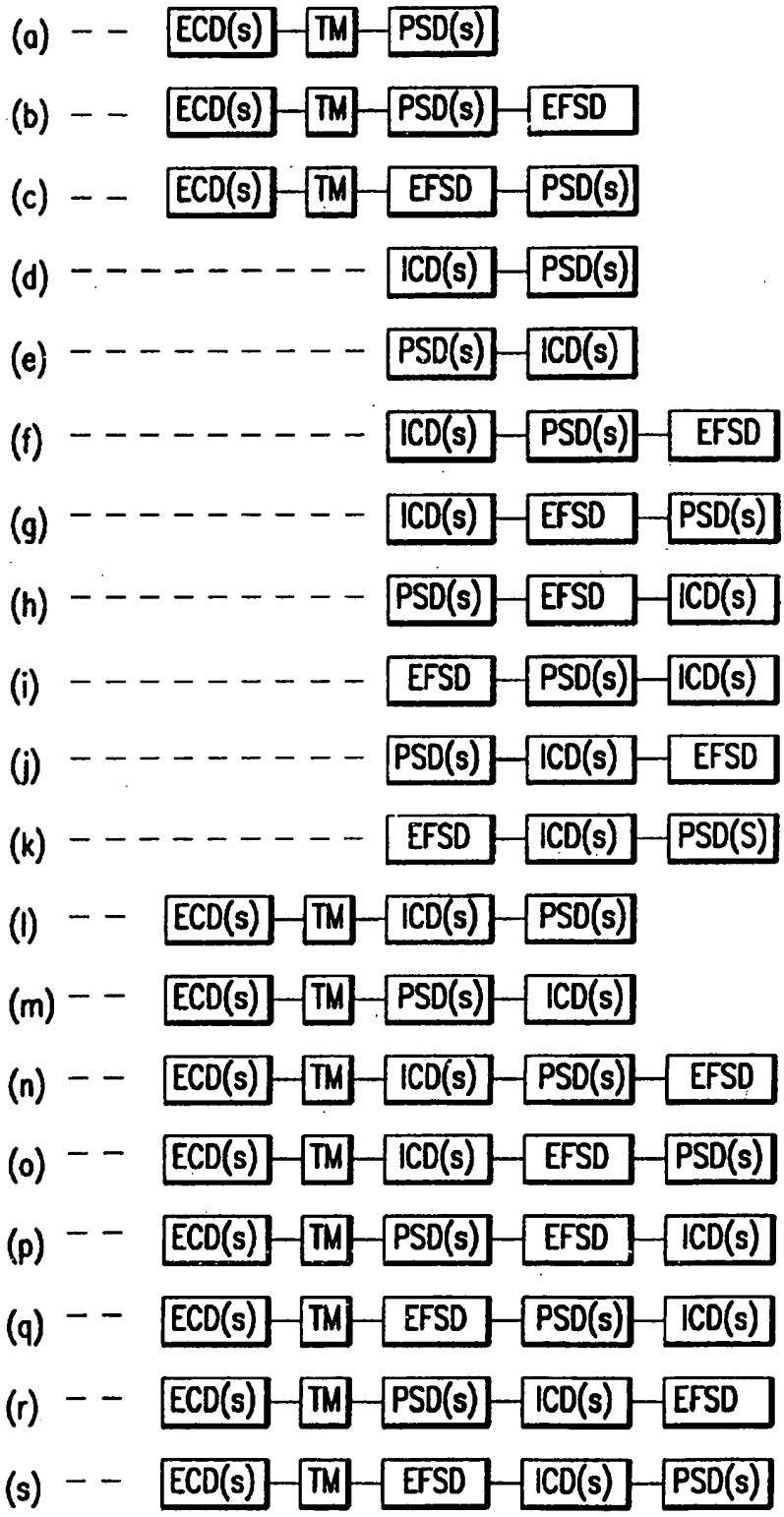
Assistant Examiner—Michael D. Pak

Attorney, Agent, or Firm—Sughrue, Mion, Zinn, Macpeak & Seas, PLLC

[57] **ABSTRACT**

The present invention is directed to novel chimeric proliferation receptor proteins and DNA sequences encoding these proteins where the chimeric proteins are characterized in three general categories. In one category, the novel chimeric proteins comprise at least three domains, namely, an extracellular inducer-responsive clustering domain capable of binding an extracellular inducer that transmits a signal to a proliferation signaling domain, a transmembrane domain and a proliferation signaling domain that signals a host cell to divide. In the second category, the novel chimeric proteins comprise at least two domains, namely, an intracellular inducer-responsive clustering domain capable of binding an intracellular inducer and a proliferation signaling domain that signals the cell to divide. In yet a third category, a novel hybrid chimeric protein receptor is contemplated that contains an intracellular or extracellular inducer domain, a transmembrane domain, a proliferation signaling domain and an effector signaling domain in a single chain molecule. Whether the binding domain is intracellular or extracellular, the binding of inducer to these novel chimeric receptor proteins induces the clustering of the binding domains to each other and further signals the cell to proliferate, and optionally, signal an effector function. The present invention further relates to expression vectors containing the nucleic acids encoding the novel chimeric receptors, cells expressing the novel chimeric receptors and therapeutic methods of using cells expressing these novel receptors for the treatment of cancer, infectious disease and autoimmune diseases, for example.

5 Claims, 6 Drawing Sheets


(a) ECD(s) TM PSD(s)
(b) ECD(s) TM PSD(s) EFSD
(c) ECD(s) TM EFSD PSD(s)
(d) ICD(s) PSD(s)
(e) PSD(s) ICD(s)
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(g) ICD(s) EFSD PSD(s)
(h) PSD(s) EFSD ICD(s)
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(l) ECD(s) TM ICD(s) PSD(s)
(m) ECD(s) TM PSD(s) ICD(s)
(n) ECD(s) TM ICD(s) PSD(s) EFSD
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(q) ECD(s) TM EFSD PSD(s) ICD(s)
(r) ECD(s) TM PSD(s) ICD(s) EFSD
(s) ECD(s) TM EFSD ICD(s) PSD(s)

FIG. 1

OLIGO 1	CCTGCTGAACTTCACTCTGTCGACACAGAAGAAGATGCC
OLIGO 2	TCGACATGCAGTATCTAAATATAAAAGAGGACTGCAATGC
OLIGO 3	CATGGCATTGCAGTCCTCTTTTATATTTAGATACTGCATG
OLIGO 4	TATGTGTCAGTGGGGCGGGCC
OLIGO 5	CGCCCCACTGACACA
OLIGO 6	GTAAGGCAGGCCATTCCCATGTCGACACAGAAGAAGATGCC
OLIGO 7	TCTGTGTCGACATGGG
OLIGO 8	TCGACATGGCACCTCCAAGTGAGGAGACACCTCTGATCCCT-CAGC
OLIGO 9	GCTGAGGGATCAGAGGTGTCTCCTCACTTGGAGGTGCCATG
OLIGO 10	GATCCCTAGTTTATTCATGGGCC
OLIGO 11	CATGAATAAACTAGG
OLIGO 12	CATCCCCCAGTGGCGCAGAGGCATGTCGACAGAGTGAAGTTC
OLIGO 13	GTCGACATGCCTCTGC
OLIGO 14	GGGCCGCCGGAATTCCATGTCGACACAGAAGAAGATGCC
OLIGO 15	TCTGTGTCGACATGGA
OLIGO 16	CCTCAACAGGGTCCTTC
OLIGO 17	GCTGATCGTCGACAACTGCAGGAACACCGG
OLIGO 18	CATCTGTGATATCTCTACACCAAGTGAGTTG
OLIGO 19	GAAGAGCAAGCGCCATGTTGAAGCCATCATTACCATTCAC

FIG. 2A

OLIGO 20 AGCCTGAAACCTGAACCCCAATCCTCTGACAGAAGAACCC

OLIGO 21 CTGGCTGGTCGACGAACGGACGATGCCCCGCATTCCCACCC-TGAAGAAC

OLIGO 22 GATTGGGGGATATCTCAGGTTTCAGGCTTTAG

OLIGO 23 GAAATCCCCTGGCTGTTAGTCGACGCGAGGGGGCAGGGCCTG

OLIGO 24 TGTTAGTCGACGCGAG

OLIGO 25 GGTCCACTCGAGATGGCCAGCAGCGGCATG

OLIGO 26 CCAGGTCCGATATCTTAGTCGACGTTCACCACGTCATAGTA

OLIGO 27 GACTGACTCTCGAGGGCGTGCAGGTGGAAACC

OLIGO 28 GACTGACTGTCGACTTCCAGTTTTAGAAGCTC

OLIGO 29 AATTCAAGGCCACAATGC

OLIGO 30 TCGAGCATTGTGGCCTTG

FIG. 2B

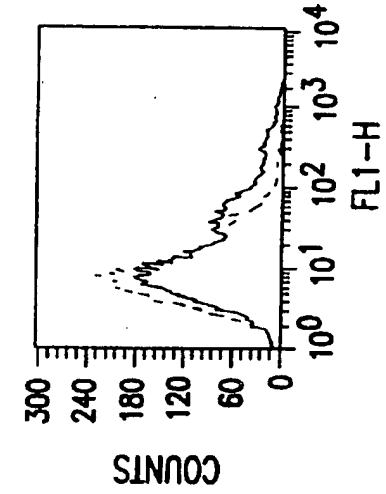

FIG.3C

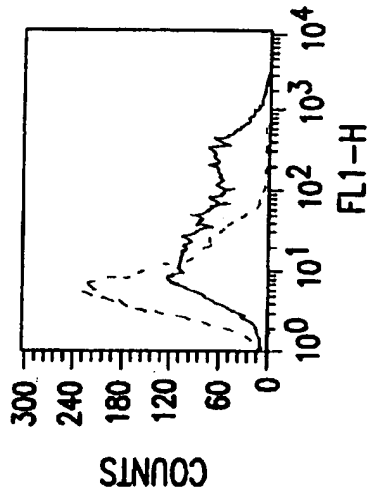

FIG.3F

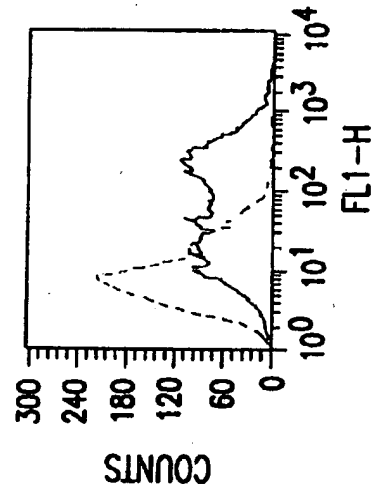

FIG.3B

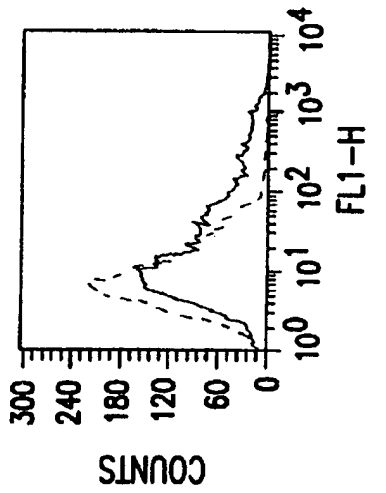

FIG.3E

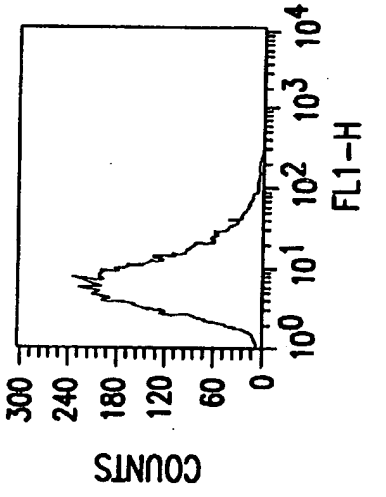

FIG.3A

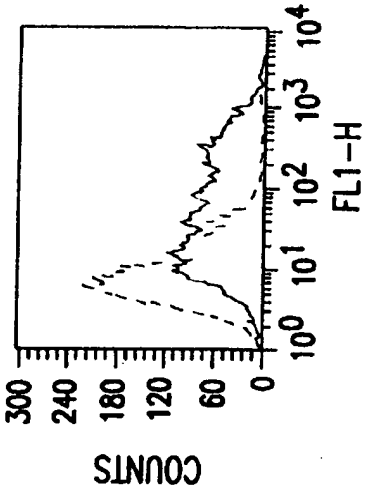

FIG.3D

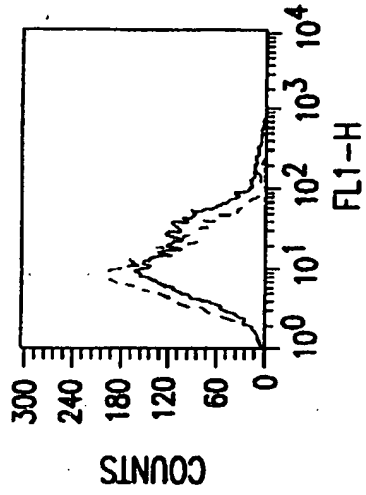

FIG.3I

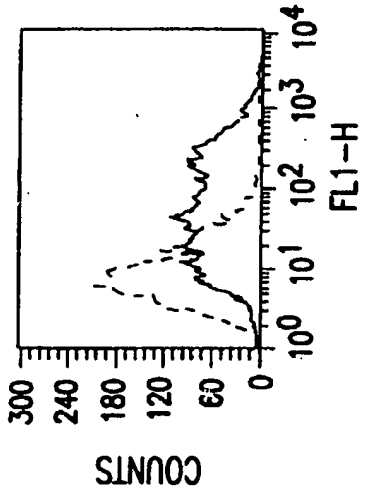

FIG.3L

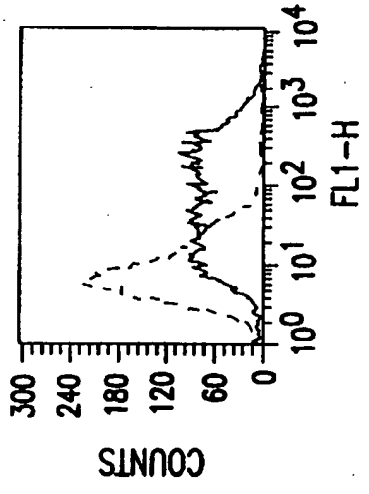

FIG.3H

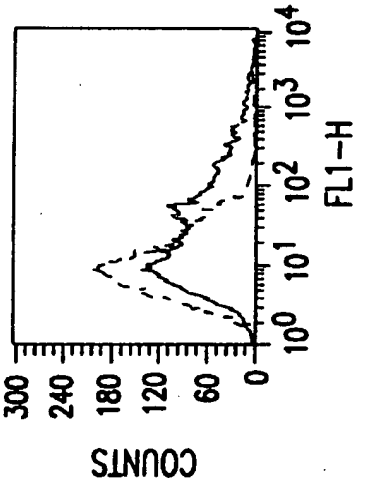

FIG.3K

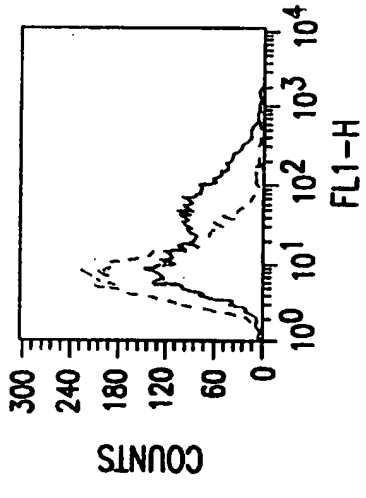

FIG.3G

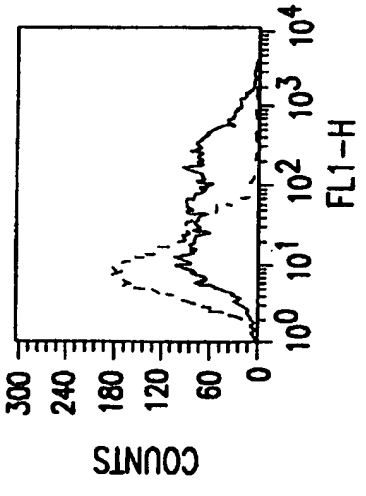

FIG.3J

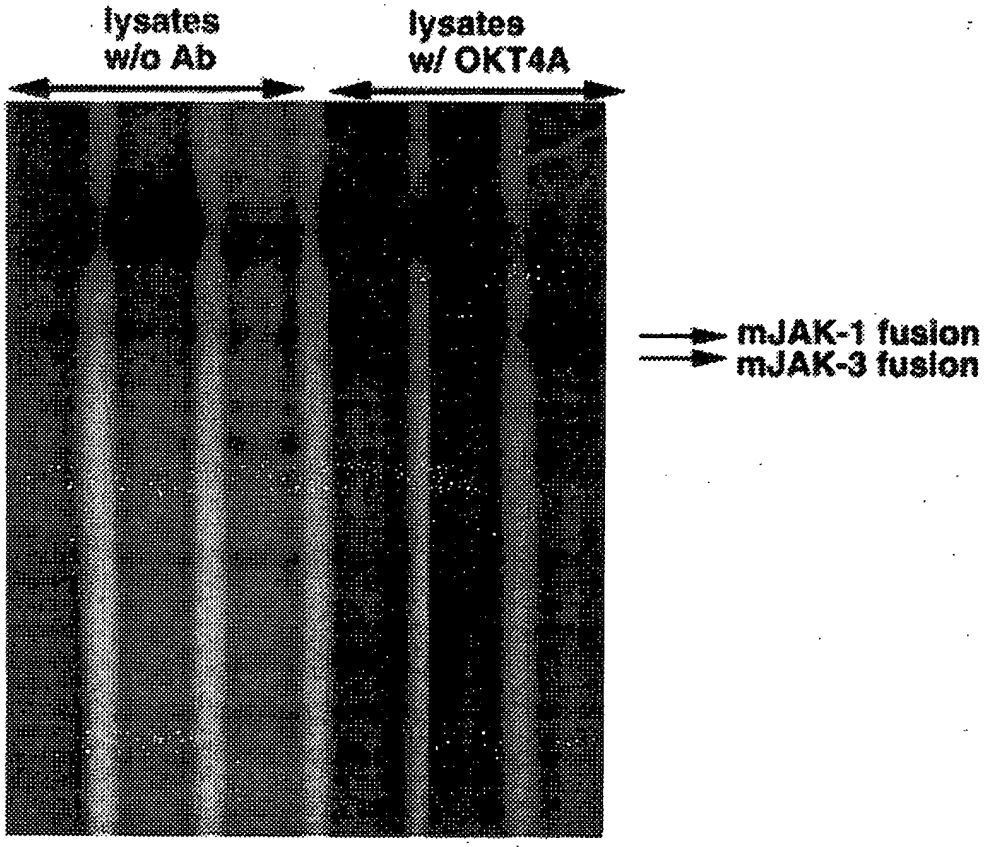

FIG.4

METHOD OF INDUCING A CELL TO PROLIFERATE USING A CHIMERIC RECEPTOR COMPRISING JANUS KINASE

This application is a continuation of application Ser. No. 08/382,846, filed Feb. 2, 1995, which is pending.

INTRODUCTION

TECHNICAL FIELD

The field of this invention relates to the construction and use of novel chimeric receptor proteins for signaling cellular proliferation and optionally, for signaling cellular effector function.

BACKGROUND

The production of novel chimeric receptor proteins which initiate signaling in a cell that results in activating a second messenger pathway in response to an inducer binding to the extracellular portion of these receptors is the subject of U.S. Pat. No. 5,359,046, the entirety of which is incorporated herein by reference. These chimeric receptor molecules comprise three domains in a single protein moiety, namely, a cytoplasmic effector function signaling domain, a transmembrane domain and an extracellular inducer binding domain. The cytoplasmic domain and extracellular domain are not naturally associated together. By mixing and matching extracellular domains with a particular type of cytoplasmic domain, one may transduce a particular signal by employing different inducers that bind to different extracellular binding domain receptors. Additionally, these single molecule receptors have the desired characteristics of binding inducer and transducing a signal without requiring the major histocompatibility complex (MHC) involvement or antigen presentation. Such characteristics make these chimeric receptors ideal in the development of cellular therapies by permitting the directed activity of cells selected for a particular effector function.

To enhance the above technology, it would be desirable to insure that cells expressing these chimeric receptors with effector function are present in the body in sufficient quantity for effective cellular therapy or treatment. This requirement may be met by the proliferation of the cells expressing the chimeric effector function receptor at the site where they would be most advantageous.

The present invention provides a strategy that consists of further engineering cells, including those expressing chimeric effector function receptors such that they are capable of proliferating in the body in an inducer molecule driven fashion and, in addition, may be growth factor independent.

There is also a general need in the field for a variety of therapeutic cells to proliferate in vivo either when they have homed to or are transplanted to the proper site or in response to an administered inducer molecule. The present invention provides a method to direct cell proliferation in this manner.

SUMMARY OF THE INVENTION

Methods involving recombinant DNA technology and recombinant protein expression are provided for the production and expression of novel chimeric receptors for regulating cellular proliferation and optionally, for signaling effector function. In one general embodiment, the novel chimeric proliferation receptor proteins comprise at least an extracellular inducer-responsive clustering domain that binds to an extracellular inducer, a transmembrane domain that crosses

the cell membrane, and a cytoplasmic proliferation signaling domain that signals the cell to divide upon the clustering of the extracellular domains. This novel chimeric proliferation receptor may optionally have an effector function signaling domain between the transmembrane domain and the proliferation signaling domain or it may be attached to the C-terminus of the proliferation signaling domain. In another general embodiment, the novel chimeric proliferation receptor proteins comprise at least an intracellular inducer-responsive clustering domain that binds to an intracellular inducer, and a cytoplasmic proliferation signaling domain that signals the cell to divide upon the clustering of the intracellular domains. This novel chimeric proliferation receptor may optionally have an effector function signaling domain attached via its N-terminus to the proliferation signaling domain or to the intracellular inducer-responsive clustering domain. Modifications of these receptors include amino acid substitutions or deletions of the domains, or the additions of one or more linker regions between various domains of these novel chimeric proliferation receptors.

The present invention also includes the preparation and expression of novel chimeric proliferation receptor proteins or modifications thereof by transducing into a host cell a DNA construct comprising a DNA fragment or variant thereof encoding the above novel chimeric proliferation receptor(s) functionally attached to regulatory sequences that permit the transcription and translation of the structural gene and expression in the host cell containing the DNA construct of interest.

The present invention further includes DNA fragments and variants thereof encoding the novel chimeric proliferation receptors including the expression vectors comprising the above DNA fragments or variants thereof, host cells transduced with the above expression vectors and methods of using the novel chimeric proliferation receptors to regulate cell growth or as therapeutics for treating cancer and infectious diseases.

DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates the structures of the chimeric proliferation receptors discussed in the detailed description.

FIGS. 2A and 2B is a listing of oligonucleotides (SEQ ID NOS:1–30) as described in the Examples, infra.

FIGS. 3 (A)–(L) are graphs of FACS analysis of CD4-Janus kinase chimeric proliferation receptor expression in 293 cells, as described in Example 10(B), infra. The dotted lines are cells stained with FITC-IgG; the solid lines are cells stained with FITC-anti-CD4. (FIG. 3(A): Mock-transfected; FIG. 3(B) CD4-ζ; FIG. 3(C) CD4-mJAK1; FIG. 3(D) CD4-ζ-mJAK1; FIG. 3(E) CD4-mJAK2; FIG. 3(F) CD4-ζ-mJAK2; FIG. 3(G) CD4-mJAK3; FIG. 3(H) CD4-ζ-mJAK3; FIG. 3(I): CD4-hJAK3; FIG. 3(J) CD4-ζ-hJAK3; FIG. 3(K): CD4-hTyk2; FIG. 3(L): CD4-ζ-hTyk2.)

FIG. 4 is an autoradiogram of immunoprecipitations of lysates from 293 cells transfected with CD4-Janus kinase constructs as described in Example 10(C). (Lanes 1 & 4: Mock-transfected; Lanes 2 & 5: CD4-mJAK1; Lanes 3 & 6: CD4-mJAK3; Lanes 1–3: no antibody and Lanes 4–6: OKT4A antibody.)

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As noted above, the present invention generally relates to novel chimeric proliferation receptor proteins and DNA sequences encoding these novel chimeric receptor proteins

which may or may not additionally contain an effector function signaling domain. The novel chimeric proliferation receptors (CPRs) provided herein may be further characterized in that the inducer binding domain of the CPR is expressed extracellularly or intracellularly. CPRs may be introduced into cells already expressing a chimeric effector function receptor previously as described in U.S. Pat. No. 5,359,046 or the two receptors may be introduced together and co-expressed in the same cell. In this aspect, the CPR containing cells of the present invention have the distinct advantage of specific expansion in response to a specific inducer molecule that may simultaneously stimulate effector function in the same expanded cell population. Alternatively, CPRs of the present invention may be introduced into cells without a chimeric effector function receptor, to allow them to proliferate in vivo. Further aspects of the present invention will be discussed in detail below following a definition of terms employed herein.

Definitions:

The term "extracellular inducer-responsive clustering domain" or "ECD" refers to the portion of a protein of the present invention which is outside of the plasma membrane of a cell and binds to at least one extracellular inducer molecule as defined below. The ECD may include the entire extracytoplasmic portion of a transmembrane protein, a cell surface or membrane associated protein, a secreted protein, a cell surface targeting protein, a cell adhesion molecule, or a normally intracytoplasmic ligand-binding domain, and truncated or modified portions thereof. In addition, after binding one or more inducer molecule(s), the ECDs will become associated with each other by dimerization or oligomerization, i.e., "cluster".

The term "intracellular inducer-responsive clustering domain" or "ICD" refers to the portion of a protein which is inside of the plasma membrane of a cell, that binds to at least one intracellular inducer molecule as defined below. After binding one or more inducer molecule(s), the ICDs will become associated with each other by dimerization or oligomerization, i.e., "cluster".

The term "proliferation signaling domain" or "PSD" refers to a protein domain which signals the cell to enter mitosis and begin cell growth. Examples include the human or mouse Janus kinases, including but not limited to, JAK1, JAK2, JAK3, Tyk2, Ptk-2, homologous members of the Janus kinase family from other mammalian or eukaryotic species, the IL-2 receptor β and/or γ chains and other subunits from the cytokine receptor superfamily of proteins that may interact with the Janus kinase family of proteins to transduce a signal, or portions, modifications or combinations thereof.

The term "transmembrane domain" or "TM" refers to the domain of the protein which crosses the plasma membrane and is derived from the inducer-binding ECD domain, the effector function signaling domain, the proliferation signaling domain or a domain associated with a totally different protein. Alternatively, the transmembrane domain may be an artificial hydrophobic amino acid sequence which spans the plasma membrane.

The term "extracellular inducer molecule" refers to a ligand or antigen which binds to and induces the clustering of an ECD as described above or portions or modifications of the extracellular inducer molecule that are still capable of binding to and inducing the clustering of an ECD. To facilitate clustering, the inducer molecule may be intrinsically bivalent or multivalent; or it may be presented to the ECD in a bivalent or multivalent form, eg., on the surface of a cell or a virus.

The term "intracellular inducer molecule" refers to a natural or synthetic ligand that can be delivered to the cytoplasm of a cell, and binds to and induces the clustering of an intracellular inducer responsive domain. To facilitate clustering, the intracellular inducer molecule may be intrinsically bivalent or multivalent.

The term "chimeric extracellular inducer-responsive proliferation receptor" or "CEPR" refers to a chimeric receptor that comprises an extracellular inducer responsive clustering domain (ECD), a transmembrane domain and a proliferation signaling domain (PSD). The ECD and PSD are not naturally found together on a single receptor protein Optionally, this chimeric receptor may also contain an effector function signaling domain as defined below.

The term "chimeric intracellular inducer-responsive proliferation receptor" or "CIPR" refers to a chimeric receptor that comprises an intracellular inducer-responsive clustering domain (ICD) and a proliferation signaling domain (PSD). The ICD and PSD are not naturally found together on a single receptor protein. Optionally, this chimeric receptor may also contain an effector function signaling domain as defined below.

The term "effector function" refers to the specialized function of a differentiated cell. Effector function of a T cell, for example, may be cytolytic activity or helper activity including the secretion of cytokines.

The term "effector function signaling domain" or "EFSD" refers to the portion of a protein which transduces the effector function signal and directs the cell to perform its specialized function. While usually the entire EFSD will be employed, in many cases it will not be necessary to use the entire chain. To the extent that a truncated portion of the EFSD may find use, such truncated portion may be used in place of the intact chain as long as it still transduces the effector function signal. Examples are the ζ chain of the T cell receptor or any of its homologs (e.g., η chain, FcεR1-γ and -β chains, MB1 chain, B29 chain, etc.), CD3 polypeptides (γ, δ and ε), syk family tyrosine kinases (Syk, ZAP 70, etc.), the src family tyrosine kinases (Lck, Fyn, Lyn, etc.) and other molecules involved in T cell signal transduction.

The term "chimeric effector function receptor" refers to a chimeric receptor that comprises an extracellular domain, transmembrane domain and cytoplasmic domain as described in U.S. Pat. No. 5,359,046 or the EFSD domain as described above. The extracellular domain serves to bind to an inducer and transmit a signal to the cytoplasmic domain which transduces an effector function signal to the cell.

The term "modifications" refers to an addition of one or more amino acids to either or both of the C- and N-terminal ends of the intracellular and extracellular inducer molecules (in the case where these are proteins) or, the ECDs, ICDs, PSDs, EFSDs, or TMs, a substitution of one or more amino acids at one or more sites throughout these proteins, a deletion of one or more amino acids within or at either or both ends of these proteins, or an insertion of one or more amino acids at one or more sites in these proteins such that the inducer molecule binding to the ICD or the ECD is retained or improved as measured by binding assays known in the art, for example, Scatchard plots, or such that the PSD, EFSD or TM domain activities are retained or improved as measured by one or more of the proliferation assays described below. In addition, modifications can be made to the intracellular and extracellular inducer molecules and to the corresponding ICDs and ECDs to create an improved receptor-ligand binding pair.

The term "variant" refers to a DNA fragment encoding an intracellular or extracellular inducer molecule, or an ECD,

ICD, PSD, EFSD or TM domain that may further contain an addition of one or more nucleotides internally or at the 5' or 3' end of the DNA fragment, a deletion of one or more nucleotides internally or at the 5' or 3' end of the DNA fragment or a substitution of one or more nucleotides internally or at the 5' or 3' end of the DNA fragment such that the inducer molecule binding to the ICD or the ECD is retained or improved as measured by binding assays known in the art, for example, Scatchard plots, or such that the PSD, EFSD or TM domain activities are retained or improved as measured by one or more of the proliferation assays described below. In addition, modifications can be made to the intracellular and extracellular inducer molecules and to the corresponding ICDs and ECDs to create an improved receptor-ligand binding pair.

The term "linker" or "linker region" refers to an oligo- or polypeptide region of from about 1 to 30 amino acids that links together any of the above described domains of the chimeric proliferation receptors defined above. The amino acid sequence is not derived from the ICDs, ECDs, EFSDs, PSDs, or TM domains. Examples of linker regions are linker 212 and linker 205 as referenced in Betzyk et al., *J. Biol. Chem.*, 265:18615–18620 (1990) and Gruber et al., *J. Immunol.*, 152:5368–5374 (1994) respectively.

In its general embodiments, the present invention relates to novel chimeric proliferation receptors, nucleic acid sequences encoding the receptors, the vectors containing the nucleic acid sequences encoding the receptors, the host cells expressing the receptors, and methods of using of the receptors in regulating cell growth. In one aspect of the present invention, a novel chimeric proliferation receptor (CPR) protein is provided containing an inducer-responsive binding domain and a proliferation signaling domain that do not naturally exist together as a single receptor protein. One novel CPR identified herein as "chimeric extracellular inducer responsive proliferation receptor" (abbreviated CEPR) is designed to be expressed in cells, which then proliferate in response to the binding of a specific extracellular inducer molecule. The three domains that comprise CEPR are: (1) an extracellular inducer-responsive clustering domain (ECD) which serves to bind to a ligand called an extracellular inducer molecule, (2) a transmembrane domain (TM), which crosses the plasma membrane and, (3) a proliferation signaling domain (PSD) that signals the host cell to divide. Optionally, the CEPRs described above may comprise multiple PSDs attached to each other (See FIG. **1**(*a*)). Each inducer molecule or group of inducer molecules is presented multivalently (eg. more than one inducer molecule in close proximity to each other on a cell surface) to the CEPR. The inducer molecules will thus bind more than one ECD, causing the ECDs to dimerize or oligomerize (i.e. cluster together). This clustering transmits a signal through the transmembrane domain to the proliferation signaling domains, which become activated.

The host cells bearing the chimeric proliferation receptors of the present invention will expand in number in response to the binding of a specific extracellular inducer molecule, to the extracellular inducer-responsive clustering domain (ECD) of the CEPR. These ECDS include but are not limited to the following types of clustering domains: a cell surface or membrane associated molecule (eg, CD4, CD8, etc.), a secreted targeting molecule (eg., Interleukin-14 (IL-14), etc.), a cell surface/secreted targeting molecule (eg, antibody (Ab), single-chain antibody (SAb), antibody fragments, etc.), a cell adhesion molecule (e.g., ICAM, LFA-1, etc.), or portions or modification thereof. In each instance, the extracellular inducer molecules bind to the extracellular domains of the CEPR which results in the dimerization or oligomerization of the extracellular inducer responsive domains and hence, the dimerization or oligomerization (i.e. "clustering") of the proliferation signaling domains results in the transduction of a signal for cell growth.

If the chimeric extracellular inducer-responsive proliferation receptor (CEPR) of the present invention is expressed in host cells already expressing the chimeric effector function receptor of U.S. Pat. No. 5,359,046 described hereinabove (for example, CD4/zeta chimeric receptor), and binds to the same inducer as the CEPR, eg. CD4, then these dual chimeric receptor expressing cells will proliferate upon addition of the same inducer that drives effector function, eg. cytotoxicity. Alternatively, the inducer that binds to the extracellular binding domain of the chimeric effector function receptor may differ from the inducer molecule that binds to the ECD of the CEPR. In this case, one may separate cell growth (proliferation) from effector function in the same cell by stimulating with different inducer molecules.

In another aspect of the present invention, a novel chimeric proliferation receptor containing the proliferation signaling domain and effector function signaling domain together in the same protein receptor is provided. In this embodiment, the chimeric receptor comprises the three domains contained in the CEPR and additionally comprises an effector function signaling domain. Thus, the extracellular inducer responsive clustering domain (ECD) of the CEPR is linked via a transmembrane domain to two signal transducing domains. One signal transducing domain mediates the effector function signal while the other signal transducing domain mediates the proliferation signal, (for example, CD4-ζ-JAK1). Either the proliferation signaling domain or the effector function signaling domain may be linked to the transmembrane domain and is further linked on its 3' end to the second signaling domain either directly or through a linker region. Optionally, more than one PSD may be attached directly, or through a linker, to each other to form a CEPR with multiple PSDs (FIG. **1**(*b*) and (*c*)). It is contemplated that the preparation of this novel chimeric proliferation/effector function chimeric receptor will activate proliferation and effector function simultaneously in a host cell upon the binding of extracellular inducer molecules to the ECD of the receptor.

In another embodiment, the present invention relates to a second general category of chimeric proliferation receptors called "chimeric intracellular inducer-responsive proliferation receptors" or "CIPRs". Cells constructed to express CIPRs proliferate in response to a specific ligand, called an intracellular inducer molecule. This proliferation receptor contains at least two domains: (1) an intracellular inducer-responsive clustering domain (ICD) which serves to bind to a ligand called an intracellular inducer molecule, and (2) a proliferation signaling domain (PSD) that signals the cell to divide (as an example, FKBP-JAK1). The two domains comprising a CIPR may be constructed such that either the ICD or the PSD is at the N-terminus of the CIPR. A linker region such as linker 212 (Betzyk et al., *J. Biol. Chem.* 265:18615–18620 (1990)) may also be inserted between the two domains that comprise CIPRs. Each inducer molecule binds two or more ICDs, causing them to dimerize or oligomerize (i.e. cluster together). This clustering of the ICDs causes the proliferation signaling domains to become activated. A transmembrane domain is not required but may be used in the construction of these novel intracellular proliferation receptors. Optionally, a myristylation-targeting domain may be linked to the N-terminus of the ICD or the PSD to allow for membrane association (Cross et al., *Mol.*

Cell. Biol., 4:1834–1842 (1984), Spencer. et al, *Science* 262:1019–1024 (1993)). An additional option may be to construct a CIPR with more than one PSD attached directly, or through a linker, to each other (FIG. 1(*d*) and (*e*). CIPRs may be used in any host cell type for which there is a desire for regulated expansion of a therapeutic cell such as in transplantation therapy, as described infra.

The host cells bearing CIPRs of the present invention will expand in number upon binding of an intracellular inducer molecule to the intracellular inducer-responsive clustering domain (ICD) of the CIPR. These inducer molecules include but are not limited to the following ligands: natural or synthetic ligands that bind to and induce the clustering of an intracellular inducer responsive domain such as immunophilins (e.g., FKBP), cyclophilins, and steroid receptors.

The CIPRs of the present invention may also be expressed in host cells previously engineered with the chimeric effector function receptor described hereinabove. Upon addition of an extracellular inducer molecule and an intracellular inducer molecule, these cells will activate the effector function (provided by signaling through the chimeric effector function receptor) and divide (provided by signaling through the CIPR). Alternatively, the inducer that binds to the extracellular binding domain of the chimeric effector function receptor may be the same inducer as the one that binds to the ICD of the CIPR if the inducer is a intracellular inducer molecule which can be delivered to the cytoplasm of the host cell. In this situation, cell growth and effector function would be activated simultaneously in the same cell upon presentation of the intracellular inducer molecule.

In another aspect of the present invention, a novel chimeric protein receptor containing a proliferation signaling domain and effector signaling domain is provided together in the same intracellular inducer-responsive receptor (FIG. 1(*f*) through (*k*)). In this embodiment, a hybrid receptor is constructed as one protein comprising the two domains described in the CIPR of the present invention, and additionally comprising an effector function signaling domain (EFSD). Thus, the intracellular inducer responsive clustering domain (ICD) is directly connected to the proliferation signaling domain (PSD) which in turn is directly attached to an effector function signaling domain (FIG. 1(*f*)). Alternatively, the ICD may be directly connected to an effector function signaling domain which in turn is directly connected to a proliferation signaling domain (FIG. 1(*g*)). In yet another conformation of the present embodiment, either the EFSD or the PSD may be associated with the membrane via a myristylation domain or a TM domain, for example. The EFSD or the PSD is attached at its C terminus to a PSD or EFSD, respectively, which in turn is attached at its C terminus to one or more ICDs (FIG. 1(*h*) and (*i*)). In addition, CIPR proliferation/effector function receptors may be constructed by linking together the following domains (N to C terminal): a membrane-associated PSD or EFSD, followed by one or more ICDs, followed by the EFSD or PSD domain, respectively, (FIG. 1(*j*) and (*k*)). It is also possible to separate one or more domains from each other in the hybrid proliferation/effector receptors of the present embodiments with a linker region such as linker 205 (Gruber et al, *J. Tmmunol.*, 152:5368–5374 (1994)). Upon introduction of these novel hybrid chimeric proliferation/) effector function receptors into cells, one may modulate the signaling of a proliferative response and effector functional response by the addition of one or more intracellular inducer molecules.

In yet another aspect of the present invention, a novel hybrid chimeric proliferation receptor containing an extracellular inducer-responsive clustering domain (ECD), an intracellular inducer-responsive clustering domain (ICD), and a proliferation signaling domain (PSD) is provided together in the same receptor protein. In this embodiment, a hybrid inducer binding receptor is constructed as one protein comprising in the N-terminal to C-terminal direction an ECD, transmembrane domain, an ICD and a proliferation signaling domain (FIG. 1(*l*)). Alternatively, a hybrid inducer binding receptor is constructed as one protein comprising in the N-terminal to C-terminal direction an ECD, transmembrane domain, PSD and an ICD (FIG. 1(*m*)). In preparing the hybrid inducer binding receptors of the present embodiment, one may separate one or more domains of each receptor with a linker. Additionally, more than one ICD and PSD may be attached directly or via a linker to each other to form multiple ICDs and PSDs. Upon introduction of these novel hybrid inducer-binding chimeric proliferation receptors into a host cell, one may modulate proliferation of the cell by either an extracellular inducer, an intracellular inducer or a combination of these two different inducer molecules.

In still another embodiment, the present invention provides a chimeric proliferation receptor described above containing an ECD, TM, ICD and PSD (N- to C-terminal) that additionally contains an effector function signaling domain (EFSD) attached at the N-terminal (FIG. 1(*o*)) or C-terminal (FIG. 1(*n*)) end of the PSD. Multiple ECDs, ICDs and/or PSDs may be used in the construction of the above receptors. Additional embodiments of hybrid CPRs containing one or more ICD(s) and ECD(s) and one or more PSD(s) and one EFSD are contemplated that comprise the following four conformations (N- to C-terminus): ECD(s), TM, PSD(s), EFSD and ICD(s) (FIG. 1(*p*)); ECD, TM, EFSD, PSD and ICD (FIG. 1(*q*)); ECD(s), TM, PSD(s), ICD(s) and EFSD (FIG. 1(*r*)); and ECD(s), TM, EFSD, ICD(s) and PSD(s) (FIG. 1(*s*)). Upon expression of these novel proliferation/effector receptors in a host cell, one may modulate proliferation and effector signaling by adding either an extracellular inducer, an intracellular inducer or a combination of these two different inducer molecules.

The proliferation signaling domains (PSDs) that comprise the chimeric proliferation receptors (CPRs) of the present invention (both CIPRs and CEPRs) may be obtained from the cytoplasmic signal-transducing domains of the cytokine/hematopoietin receptor superfamily. The members of this mammalian receptor superfamily can transduce proliferative signals in a wide variety of cell types. These receptors are structurally related to each other. The cytoplasmic domains of the signal-transducing subunits may contain conserved motifs that are critical for transduction of proliferative signals (Bazan, *Current Biology*, 3:603–606 (1993); Boulay and Paul, *Current Biology*, 3:573–581 (1993); Wells, *Current Opinion in Cell Biology*, 6:163–173 (1994); Sato and Miyajima, *Current Opinion in Cell Biology*, 6:174–179 (1994); Stahl and Yancopoulos, *Cell*, 74:587–590 (1993); Minami et al., *Ann. Rev. Immunol.*, 11:245–267 (1993); Kishimoto et al., *Cell*, 76:253–262 (1994)). In contrast to the growth factor receptors previously described in chimeric receptors (Schlessinger and Ullrich , *Cell*, 61:203–212 (1990), Ullrich and Schlessinger, *Neuron*, 9:383–391 (1992)), the cytoplasmic portions of the cytokine receptor superfamily proteins that comprise the PSDs employed in the present invention do not contain any kinase domains or other sequences with recognizable catalytic function. Further, although the growth factor receptors described by Ullrich and the cytokine receptors employed in the present invention both dimerize upon binding of inducer, the dimerized growth factor receptors activate their intrinsic receptor

kinase activity, while the dimerized cytokine receptors employed in the present invention stimulate the activity of associated tyrosine kinases (Kishimoto et al., *Cell*, 76:253–262 (1994)). The signal-transducing components of the cytokine receptors to be used in the PSDs of the present invention include, but are not limited to, Interleukin-2 receptor β (IL-2Rβ), IL-2Rγ, IL-3Rβ, IL-4R, IL-5Rα, IL-5Rβ, IL-6R, IL-6R gp130, IL-7R, IL-9R, IL-12R, IL-13R, IL-15R, EPO-R (erythropoietin receptor), G-CSFR (granulocyte colony stimulating factor receptor), GM-CSFRα (granulocyte macrophage colony stimulating factor receptor α), GM-CSFRβ, LIFRα (leukemia inhibitory factor receptor α), GHR (growth hormone receptor), PRLR (prolactin receptor), CNTFR (ciliary neurotrophic factor receptor), OSMR (oncostatin M receptor) IFNRα/β (interferon α/β receptor), IFNRγ, TFR (tissue factor receptor),and TPOR (thrombopoietin or mpl-ligand receptor)(Minami et al., *J. Immunol.*, 152:5680–5690 (1994); Boulay and Paul, *Current Biology*, 3:573–581 (1993); Wells, *Current Opinion in Cell Biology*, 6:163–173 (1994)).

The IL-2, IL-3 and IL-6 subfamilies of the above cytokine receptor superfamily, which are active in many different cell types, may supply the PSDs of the CPRs of the present invention. The IL-2 receptor subfamily includes, but is not to be limited to, the receptors for IL-2, IL-4, IL-7, IL-9, IL-13 and IL-15. IL-2R, IL-4R, IL-7R, IL-9R, IL-13R and IL-15R share IL-2Rγ, one of the signal transducing components of the IL-2R (Noguchi et al., *Science*, 262:1877–1880 (1993); Russel et al., *Science*, 262:1880–1884 (1993); Minami et al., *J. Immunol.*, 152:5680–5690 (1994)). IL-2R and IL-15R share a second transducing component, IL-2Rβ (Giri et al., *EMBO J.*, 13:2822–2830 (1994)). These cytokines act on a wide variety of cell types, for example, B cells, T cells including LAK cells and thymocytes, NK cells, and oligodendroglial cells (Kishimoto et al., *Cell*, 76:253–262 (1994)). In addition, high affinity receptors to IL-15 are found on myeloid cells, vascular endothelial cells, and on stromal cells types from bone marrow, fetal liver and thymic epithelium (Giri et al., *EMBO J.*, 13:2822–2830 (1994)). The IL-3 receptor subfamily includes, but is not limited to, the receptors for IL-3, IL-5 and GM-CSF (Sato and Miyajima, *Current Opinion in Cell Bioloay*, 6:174–179 (1994)). These cytokine receptors contain a common signal-transducing, or β chain which has a large cytoplasmic domain whose membrane proximal region is critical for c-myc induction and proliferative signaling activity (Quelle et al., *Mol. Cell. Biol.*, 14:4335–4341 (1994)). This family of cytokines act on overlapping cell types during hematopoiesis including blast cells, granulocytes, macrophages, monocytes and eosinophils (Kishimoto et al., *Cell*, 76:253–262 (1994)). The IL-6 receptor subfamily includes, but is not limited to, the receptors for IL-6, CNTF, LIF, OSM, IL-11, G-CSFR and IL-12. IL-6R, CNTFR, LIFR and OSMR have a common signal-transducing chain (gp130) with a cytoplasmic domain whose membrane proximal region is critical for signaling activity (Sato and Miyajima, *Current Opinion in Cell Biology*, 6:174–179 (1994), Narazaki et al., *Proc. Natl. Acad. Sci.*, 91:2285–2289 (1994)). These cytokines act on a wide variety of cell types, including ciliary, sympathetic, sensory and motor neurons, embryonic stem cells, control of the differentiation of B cells, plasmacytomas, megakaryocytes, myeloid cells, osteoclasts, and hepatocytes (Kishimoto et al., *Cell*, 76:253–262 (1994)). Other members of the cytokine receptor superfamily which may be a part of the above subfamilies, or may be members of novel subfamilies include the receptors for EPO, TPO, GH and PRL, which are also found on many cell types (Wells, *Current Opinion in Cell Biology*, 6:163–173 (1994), Stahl and Yancopoulos, *Cell*, 74:587–590 (1993)). The more distantly related IFNα/β and IFNγ receptors, found in most cell types also contain cytoplasmic domains of related structure (Farrar and Schreiber, *Annu. Rev. Immunol.*, 11:571–611 (1993), Taga and Kishimoto, *FASEB J.*, 6:3387–3396 (1992)).

The proliferation signaling domains employed in constructing the CPRs of the present invention may also be obtained from any member of the Janus or JAK eukaryotic family of tyrosine kinases, including Tyk2, JAK1, JAK2, JAK3 and Ptk-2. Members of the Janus kinase family are found in all cell types. They associate with various signal transducing components of the cytokine receptor superfamily discussed above and respond to the binding of extracellular inducer by the phosphorylation of tyrosines on cytoplasmic substrates (Stahl and Yancopoulos, *Cell*, 74:587–590 (1993)). They are thus an integral part of the control of cell proliferation in many different kinds of cells. The members of this family are marked by similar multidomain structures and a high degree of sequence conservation. Unique among tyrosine kinases, the Janus kinase family may have two non-identical tandem kinase-like domains, only one of which may have catalytic activity (Firmbach-Kraft et al., *Oncopene*, 5:1329–1336 (1990); Wilks et al., *Mol. Cell. Biol.*, 11:2057–2065 (1991); Harpur et al., *Oncogene*, 7:1347–1353 (1992)). The Janus kinases used in the present invention, unlike the src kinases, do not have src homology sequences (SH2, SH3) or a consensus sequence for myristylation. Unlike the receptor tyrosine kinases (RTK), the Janus kinases are not membrane proteins and do not contain transmembrane spanning domains (Ullrich and Schlessinger, *Neuron*, 9:383–391 (1992)). The kinase activity of the Janus kinases is usually activated after the binding of inducers to their associated cytokine family receptors and the oligomerization of the receptors (Stahl and Yancopoulos, *Cell*, 74:587–590 (1993)). This activation, in turn, triggers the initiation of intracellular signaling cascades.

JAK3 can be employed as a PSD in any of the CPRs of the present invention. Its activation by IL-2 parallels c-myc induction and the onset of DNA synthesis. JAK3 is involved with IL-2, IL-4 and IL-7 induced stimulation of T, NK and myeloid cells (Witthuhn et al., *Nature*, 370:153–157 (1994); Russell et al., *Science*, 366:1042–1044 (1994); Kawamura et al., *Proc. Natl. Acad. Sci.*, 91:6374–6378 (1994); Miyazaki et al., *Science*, 266:1045–1047 (1994); Johnston et al., *Nature*, 370:151–153 (1994); Asao et al., *FEBS Letters*, 351:201–206 (1994), Zeng et al., *FEBS Letters*, 353:289–293 (1994)). JAK2, a component of growth factor signaling in a wider variety of cells, can also be used in the CPRs of the present invention. It is activated by EPO, GH, prolactin, IL-3, GM-CSF, G-CSF, IFNγ, LIF, OSM, IL-12 and IL-6 (Watling et al., *Nature*, 366:166–170 (1993); Witthuhn et al., *Cell*, 74:227–236 (1993); Argetsinger et al., *Cell*, 74:237–244 (1993); Stahl et al., *Science*, 263:92–95 (1994); Narazaki et al., *Proc. Natl. Acad. Sci.*, 91:2285–2289(1994); Quelle et al., *Mol. Cell. Biol.*, 14:4335–4341 (1994); Silvennoinen et al., *Nature*, 366:583–585 (1993); Darnell et al., *Science*, 264:1415–1421 (1994)Campbell et al, *Proc. Natl. Acad. Sci.*, 91:5232–5236 (1994), Bacon et al., *J. Exp. Med.*, 181:399–404 (1995); (Harpur *Oncogene* 7: 1347–1353, 1992)). The present invention also contemplates the use of JAK1 as a PSD in the present invention. Its activity is also promiscuous, being an integral part of IFNR-α, IFNR-γ, IL-2Rβ, IL-6R and

CNTFR signaling (Muller et al., *Nature*, 366:129–135 (1993); Silvennoinen et al., *Nature*, 366:583–585 (1993); Stahl et al., *Science*, 263:92–95 (1994), Tanaka et al., *Proc. Natl. Acad. Sci.*, 91:7271–7275 (1994)). Tyk2, which may also be employed as a PSD, is involved with IFN-α, IL-6, IL-12, and CNTF induced signaling (Velazquez et al., *Cell*, 70:313–322 (1992); Silvennoinen et al., *Nature*, 366:583–585 (1993); Stahl et al., *Science*, 263:92–95 (1994); Colamonici et al., *J. Biol. Chem.*, 269:3518–3522 (1994); Darnell et al., *Science*, 264:1415–1421 (1994), Bacon et al., *J. Exp. Med.*, 181:399–404 (1995)) and is found in both hematopoietic and non-hematopoietic tissues (Firmbach-Kraft et al., *Oncogene* 5: 1329–1336, 1990). In addition to the Janus kinases described above, a new JAK kinase Ptk-2 has recently been described in embryonic hippocampal neurons (Sanchez et al. *Proc. Natl. Acad. Sci.*, 91:1819–1823 (1994), and can be used to form the proliferation signaling domain of any of the chimeric proliferation receptor proteins of the present invention.

One may introduce the CPR into cells where the PSD being used is not naturally found in those cells or is part of a pathway which is ordinarily not active in those cells. This unnatural expression of a particular Janus kinase or cytokine receptor subunit may have added utility. For example, if the PSDs are more active in this unnatural location, they may be more efficient stimulators of proliferation. Alternatively, if the PSDs are less active in the unnatural location they may be less likely to be constitutively active and thus more responsive to an inducer.

The transmembrane domain may be contributed by the protein contributing the proliferation signaling portion, the protein contributing the extracellular inducer clustering domain, or by a totally different protein. For the most part it will be convenient to have the transmembrane domain naturally associated with one or the other of the other domains. In some cases it will be desirable to employ the transmembrane domain of the ζ, η or FcεR1γ chains or related proteins which contain a cysteine residue capable of disulfide bonding, so that the resulting chimeric protein will be able to form disulfide linked dimers with itself, or with unmodified versions of the ζ, η or FcεR1γ chains or related proteins. In some instances, the transmembrane domain will be selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex. In other cases it will be desirable to employ the transmembrane domain of ζ, η, FcεR1-γ and -β, MB1 (Ig α), B29 (Igβ), Bovine Leukemia Virus gp30 (BLV gp30), or CD3-γ, δ, or ε, in order to retain physical association with other members of the receptor complex.

The CPRs of the present invention may be designed so as to avoid interaction with other surface membrane proteins native to the target host. In order to achieve this, one may select for a transmembrane domain which is known not to bind to other transmembrane domains, or one may modify specific amino acids, e.g. substitute for a cysteine, or the like.

The extracellular inducer-responsive clustering domain (ECD) may be obtained from any of the wide variety of extracellular domains of eukaryotic transmembrane proteins, secreted proteins or other proteins associated with ligand binding and/or signal transduction. The ECD may be part of a protein which is monomeric, homodimeric, heterodimeric, or associated with a larger number of proteins in a non-covalent or disulfide-bonded complex.

In particular, the ECDs may consist of monomeric or dimeric immunoglobulin molecules, or portions or modifications thereof, which are prepared in the following manner.

The full-length IgG heavy chain comprising the VH, CH1, hinge, and the CH2 and CH3 (Fc) Ig domains is fused to the proliferation signaling domain (PSD) via the appropriate transmembrane domain. If the VH domain alone is sufficient to confer antigen-specificity (so-called "single-domain antibodies"), homodimer formation of the Ig-PSD chimera is expected to be functionally bivalent with regard to antigen binding sites. If both the VH domain and the VL domain are necessary to generate a fully active antigen-binding site, both the IgH-PSD molecule and the full-length IgL chain are introduced into cells to generate an active antigen-binding site. Dimer formation resulting from the intermolecular Fc/hinge disulfide bonds results in the assembly of Ig-PSD receptors with extracellular domains resembling those of IgG antibodies. Derivatives of this Ig-PSD chimeric receptor include those in which only portions of the heavy chain are employed in the fusion. For example, the VH domain (and the CH1 domain) of the heavy chain can be retained in the extracellular domain of the Ig-PSD chimera (VH-PSD), but VH-PSD dimers are not formed. As above, the full-length IgL chain can be introduced into cells to generate an active antigen-binding site.

As indicated, the ECD may consist of an Ig heavy chain which may in turn be covalently associated with Ig light chain by virtue of the presence of the CH1 region, or may become covalently associated with other Ig heavy/light chain complexes by virtue of the presence of hinge, CH2 and CH3 domains. The two heavy/light chain complexes may have different specificities, thus creating a CPR which binds two distinct antigens. Depending on the function of the antibody, the desired structure and the signal transduction, the entire chain may be used or a truncated chain may be used, where all or a part of the CH1, CH2, or CH3 domains may be removed or all or part of the hinge region may be removed.

Because association of both the heavy and light V domains are required to generate a functional antigen binding site of high affinity, in order to generate a Ig chimeric receptor with the potential to bind antigen, a total of two molecules will typically need to be introduced into the host cell. Therefore, an alternative and preferred strategy is to introduce a single molecule bearing a functional antigen binding site. This avoids the technical difficulties that may attend the introduction and coordinated expression of more than one gene construct into host cells. This "single-chain antibody" (SAb) is created by fusing together the variable domains of the heavy and light chains using an oligo- or polypeptide linker, thereby reconstituting an antigen binding site on a single molecule.

Single-chain antibody variable fragments (SAbFv) in which the C-terminus of one variable domain (VH or VL) is tethered to the N-terminus of the other (VL or VH, respectively), via a oligo- or polypeptide linker, have been developed without significantly disrupting antigen binding or specificity of the binding (Bedzyk et al. (1990) *J. Biol. Chem.*, 265:18615; Chaudhary et al. (1990) *Proc. Natl. Acad. Sci.*, 87:9491). The SAbFvs used in the present invention may be of two types depending on the relative order of the VH and VL domains: VH-l-VL or VL-l-VH (where "l" represents the linker). These SAbFvs lack the constant regions (Fc) present in the heavy and light chains of the native antibody. In another aspect of the present invention, the SAbFv fragment may be fused to all or a portion of the constant domains of the heavy chain, and the resulting ECD is joined to the PSD via an appropriate transmembrane domain that will permit expression in the host cell. The resulting CPRs differ from the SAbFvs,

described above, in that upon binding of antigen they initiate signal transduction via their cytoplasmic domain.

To aid in the proper folding and efficient expression of the CPRs, the antibody-derived ECDs may be connected at their C-terminal end to one of a number of membrane hinge regions which are a normal part of membrane-bound immunoglobulin molecules. For example, the eighteen amino acids of the IGHG3 M1 exon may be used (Bensmana and Lefranc, *Immunogenet.*, 32:321–330 (1990)). The TM domain is attached to the C-terminal end of the membrane hinge. It is also contemplated that membrane hinge sequences may be used to connect non-antibody derived ECDs to the transmembrane domains to increase CPR expression.

Diabodies may also be used as ECDs in the present invention. Diabodies contain two chimeric immunoglobulin chains, one of which comprises a VH domain connected to a VL domain on the same polypeptide chain (VH-VL). A linker that is too short to allow pairing of the VH and VL domains on this chain with each other is used so that the domains will pair with the complementary VH and VL domains on the other chimeric immunoglobulin chain to create two antigen-binding sites (Holliger et al., *Proc. Natl. Acad. Sci.* 90:6444–6448 (1993)). As described above, one of these chains is linked to the membrane hinge and/or the TM domain, which in turn is linked to the PSD and/or ESD. The other chain (not connected to a PSD) will be co-expressed in the same cell to create a CPR with a diabody ECD which will respond to two different extracellular inducer molecules.

Various naturally occurring receptors may also be employed as ECDs, where the receptors are surface membrane proteins, including cell differentiation antigens such as CD4 and CD8, cytokine or hormone receptors or cell adhesion molecules. The receptor may be responsive to a natural ligand, an antibody or fragment thereof, a synthetic molecule, e.g., drug, or any other agent which is capable of inducing a signal. In addition, either member of a inducer/receptor pair, where one is expressed on a target cell such as a cancer cell, a virally infected cell or an autoimmune disease causing cell, may also be used as an ECD in the present invention. In addition, the receptor-binding domains of soluble protein ligands or portions thereof could be employed as ECDs in the CPRs of the present invention. In addition, for example, binding portions of antibodies, cytokines, hormones, or serum proteins can be used. In addition, the soluble components of the cytokine receptors such as IL-6R, IL-4R, and IL-7R can be used (Boulay and Paul *Current Biology* 3: 573–581, (1993)).

"Hybrid" ECDs can also be used in the present invention. For example, two or more antigen-binding domains from antibodies of different specificities, two or more different ligand-binding domains, or a combination of these domains can be connected to each other by oligo- or polypeptide linkers to create multispecific extracellular binding domains. These ECDs can be used to create CPRs of the present invention which will respond to two or more different extracellular inducer molecules. (See FIG. 1(*a*)–(*c*) and (1)–(*s*) that illustrate the above embodiment).

Where a receptor is a molecular complex of proteins, where only one chain has the major role of binding to the ligand, it will usually be desirable to use solely the extracellular portion of the ligand binding protein. Where the extracellular portion may complex with other extracellular portions of other proteins or form covalent bonding through disulfide linkages, one may also provide for the formation of such dimeric or multimeric extracellular regions. Also, where the entire extracellular region is not required, truncated portions thereof may be employed, where such truncated portion is functional. In particular, when the extracellular region of CD4 is employed, one may use only those sequences required for binding of gp120, the HIV envelope glycoprotein. In the case in which Ig is used as the extracellular region, one may simply use the antigen binding regions of the antibody molecule and dispense with the constant regions of the molecule (for example, the Fc region consisting of the CH2 and CH3 domains).

In some instances, a few amino acids at the joining region of the natural protein domain may be deleted, usually not more than 30, more usually not more than 20. Also, one may wish to introduce a small number of amino acids at the borders, usually not more than 30, more usually not more than 20. The deletion or insertion of amino acids will usually be as a result of the needs of the construction, providing for convenient restriction sites, ease of manipulation, improvement in levels of expression, proper folding of the molecule or the like. In addition, one may wish to substitute one or more amino acids with a different amino acid for similar reasons, usually not substituting more than about five amino acids in any one domain. The PSD, ECD, EFSD and ICD will generally be from about 50 to 1500 amino acids, depending upon the particular domain employed, while the transmembrane domain will generally have from about 20 to 35 amino acids.

Normally, the signal sequence at the 5' terminus of the open reading frame (ORF) which directs the chimeric protein to the surface membrane will be the signal sequence of the ECD. However, in some instances, one may wish to exchange this sequence for a different signal sequence. However, since the signal sequence will be removed from the protein during processing, the particular signal sequence will normally not be critical to the subject invention.

Extracellular inducers of the present invention can be antigens which bind the ECDs, described above. These may include viral proteins, (e.g. gp120 and gp41 envelope proteins of HIV, envelope proteins from the Hepatitis B and C viruses, the gB and other envelope glycoproteins of human cytomegalovirus, the envelope proteins from the Kaposi's sarcoma-associated herpesvirus), and surface proteins found on cancer cells in a specific or amplified fashion, (eg the IL-14 receptor, CD19 and CD20 for B cell lymphoma, the Lewis Y and CEA antigens for a variety of carcinomas, the Tag72 antigen for breast and colorectal cancer, EGF-R for lung cancer, and the HER-2 protein which is often amplified in human breast and ovarian carcinomas). For other receptors, the receptors and ligands of particular interest are CD4, where the ligand is the HIV gp120 envelope glycoprotein, and other viral receptors, for example ICAM, which is the receptor for the human rhinovirus, and the related receptor molecule for poliovirus.

The intracellular clustering domain (ICD) can be obtained from the inducer binding domains of a variety of intracellular proteins. For example, eukaryotic steroid receptor molecules can be used as ICDs (e.g. the receptors for estrogen, progesterone, androgens, glucocorticoids, thyroid hormone, vitamin D, retinoic acid, 9-cis retinoic acid and ecdysone). In addition, variants of steroid and other receptors which fail to bind their native inducer, but still bind to an antagonist, can be prepared by one skilled in the art and used to make the CPRs of this invention. For example, a C-terminal deletion mutant of the human progesterone receptor, which fails to bind progesterone, can be clustered by the addition of progesterone antagonists, including RU 486 (Wang et al., *Proc Natl Acad Sci* 91: 8180–8184, 1994).

Binding domains from the eukaryotic immunophilin family of molecules may also be used as ICDs. Examples include but are not limited to members of the cyclophilin family: mammalian cyclophilin A, B and C, yeast cyclophilins 1 and 2, Drosophila cyclophilin analogs such as ninaA; and members of the FKPB family: the various mammalian isoforms of FKBP and the FKBP analog from Neurospora (Schreiber, *Science*, 251:283–287 (1991), McKeon, *Cell*, 66:823–826, (1991), Friedman and Weissman, *Cell*, 66:799–806, (1991), Liu et al., *Cell*, 66:807–815 (1991)). For example, the inducer binding portion of the immunophilin, FKBP12, which can be clustered in the cytoplasm by the addition of FK1012, a synthetic dimeric form of the immunosuppressant FK506 (Spencer et al., *Science* 262: 1019–1024 (1993) can be used as an ICD.

The intracellular inducers of the present invention must be molecules which can be delivered to the cytoplasm. For example, the inducer may be lipophilic, or be transported into the cell by active transport or pinocytosis, by fusion with a liposome carrying the inducer, or by semi-permeabilization of the cell membrane. The intracellular inducers cluster the ICDs which make up the CIPRs of the present invention. Examples of inducers include, but are not limited to synthetic dimeric molecules such as FK1012 (Spencer et al., *Science*, 262:1019–1024 (1993)) or dimeric derivatives of the binding domains of other immunophilin binding molecules such as cyclosporin, rapamycin and 506BD (Schreiber, *Science*, 251:283–287 (1991), McKeon, *Cell*, 66:823–826, (1991)). Steroids, such as estrogen, progesterone, the androgens, glucocorticoids, thyroid hormone, vitamin D, retinoic acid, 9-cis retinoic acid or ecdysone, or antagonists or derivatives of these molecules may also be used as intracellular inducer molecules. In particular the steroid antagonist RU 486 may be used (Wang et al., *Proc. Natl. Acad. Sci.*, 91:8180–8184 (1994)).

The effector function signaling domains (EFSDs) employed in the present invention may be derived from a protein which is known to activate various second messenger pathways. One pathway of interest is that involving phosphatidylinositol-specific phospholipase hydrolysis of phosphatidylinositol-4,5-biphosphate, and production of inositol-1,4,5-trisphosphate and diacylglycerol. The calcium mediated pathway, the tyrosine and serine/threonine kinase and phosphatase pathway, the adenylate cyclase, and the guanylate cyclase pathways may also be second messenger pathways. EFSDs of interest include proteins with ARAM motifs (Reth, *Nature*, 338:383–384 (1989), Weiss, *Cell*, 73:209–212, (1993)), for example, the ζ chain of the T-cell receptor, the η chain, which differs from the ζ chain only in its most C-terminal exon as a result of alternative splicing of the ζ mRNA, the γ and β subunits of the FcϵR1 receptor, the MB1 (Igα) and B29 (Igβ) chains of the B cell receptor, the BLV gp30 protein and the δ, γ, and ϵ chains of the T-cell receptor (CD3 chains), other protein homologous to the above protein subunits including synthetic polypeptides with ARAM motifs, and such other cytoplasmic regions which are capable of transmitting a signal as a result of interacting with other proteins capable of binding to a inducer (Romeo et al., *Cell*, 68:889–897 (1992); Weiss, *Cell*, 73:209–212 (1993)). The syk family of tyrosine kinases may also be used as effector function signaling domains. The clustering of these domains from Syk and ZAP-70 leads to the activation of T cell cytolytic activity (Kolanus et al., *Cell*, 74:171–183 (1993)). In addition, the src family of tyrosine kinases (Lck, Fyn, Lyn, etc.(Rudd et al., *Immunology Today*, 15:225–234 (1994)) and molecules involved in T cell transduction may be used as EFSDs in the present invention. A number of EFSDs or functional fragments or mutants thereof may be employed, generally ranging from about 50 to 1500 amino acids each, where the entire naturally occurring cytoplasmic region may be employed or only an active portion thereof.

The CPRs of the present invention are employed in a wide variety of target host cells, normally cells from vertebrates, more particularly, mammals, desirably domestic animals or primates, particularly humans. In particular, the subject invention may also find application in the expansion of lymphoid cells, e.g., T lymphocytes, B lymphocytes, cytotoxic lymphocytes (CTL), natural killer cells (NK), tumor-infiltrating-lymphocytes (TIL) or other cells which are capable of killing target cells when activated. In addition, suitable host cells to introduce CPRs of the present invention include hematopoietic stem cells, which develop into cytotoxic effector cells with both myeloid and lymphoid phenotype including granulocytes, mast cells, basophils, macrophages, natural killer (NK) cells and T and B lymphocytes. In particular, diseased cells, such as cells infected with HIV, HTLV-I or II, cytomegalovirus, hepatitis B or C virus, *Mycobacterium avium*, etc., neoplastic cells, or autoimmune disease-causing cells where the diseased cells have a surface marker associated with the diseased state may be made specific targets of the cells expressing the CPRs of the present invention. In the present invention, a cell may express dual CEFR and CPR receptors, which contain the same extracellular binding domain (eg. CD4), or a cell may express a hybrid chimeric receptor combining both signaling domains (EFSD and PSD). In each case, the binding of one inducer to the extracellular binding domain will stimulate cells to act as therapeutic agents at the same time they are expanding in response to binding to inducer, e.g., gp120 for HIV or cancer-specific antigens.

In a preferred embodiment, the present invention relates to the design of chimeric proliferation receptor (CPR) molecules which can endow T cells with the ability to proliferate in an antigen-specific and IL-2 independent manner. A T cell ordinarily requires as many as three distinct stimuli to become fully activated and begin to proliferate. It must receive two signals from the antigen presenting cell (APC). The first of these signals occurs upon engagement of the T cell antigen receptor with the peptide antigen-MHC complex. The second costimulatory signal is provided through the interaction of the CD28 or CTLA4 proteins on the T cell surface with either the B7-2 or B7 proteins, their counter-receptors on the APC (Clark and Ledbetter, *Nature*, 367:425–428 (1994); Croft, *Current Opinion in Immunology*, 6:431–437 (1994)). In addition to these two signals provided during cell to cell contact between the T cell and APC, it is apparent that certain cytokines, for example IL-2, play an important role in initiating and sustaining ongoing proliferation of activated T cells (Taniguchi and Minami, *Cell*, 73:5–8 (1993)). The antigen receptor-mediated signal (e.g., anti-CD3 MAb) and the costimulatory signal (e.g., APC) play an important role in initiating and sustaining T cell proliferation, for example, by inducing IL-2 receptors which will in turn make the T cell responsive to autocrine or exogenous IL-2 stimulation. Chimeric proliferation receptors for T cells can route an antigen signal directly through the IL-2 signaling apparatus, and bypass the need to engage the T cell receptor and costimulatory receptor to elicit T cell proliferation, while still maintaining antigen specificity. This chimeric receptor will link an ECD which is an antigen binding moiety such as an antibody or a viral receptor (e.g., CD4, the receptor for HIV) to a proliferation signaling domain which is a com-

ponent of the IL-2R. One embodiment of the CPR invention would be to use one of the subunits of the IL-2 receptor (IL-2R) as a proliferation signaling domain. Specifically, the β and γ chains of the IL-2R may be utilized as PSDs in the present invention. Alternatively, the CPRs may incorporate both of all or part of the transducing domains of the IL-2Rβ and γ, which are connected through the use of an appropriate polypeptide linker sequence, in a single chimeric receptor. In a further embodiment, the CPR containing the IL-2Rβ PSD or the IL-2Rγ PSD alone is complemented with the native form of IL-2R γ or IL-2Rβ subunit respectively, which is provided by transduction. It is further contemplated that the signal transducing domains of the cytokine receptor superfamily described above may function as the PSDs in the CPRs in T cells of the present invention. In a further embodiment, chimeric proliferation receptors may incorporate more than one signaling domain chosen from the cytokine receptor family, which may be connected through an appropriate oligo- or polypeptide linker sequence in a single chimeric receptor.

In another preferred embodiment, the present invention relates to the use of chimeric proliferation receptors to induce the proliferation of T cells, where the proliferation signaling domains are comprised of one or more of the family of Janus kinases, i.e., JAK1, JAK2, JAK3, Tyk2 and Ptk-2. In the most preferred embodiment, either JAK1 or JAK3 alone or together may be employed as the PSD(s) since they play a critical role in IL-2 induced proliferation of T cells: The kinase activity of both JAK1 and JAK3 becomes stimulated after IL-2 binding to the IL2R. JAK1 and JAK3 are associated with the membrane proximal regions of the IL-2Rβ and γ chains, respectively, which are integral to the transmission of proliferative stimuli (Asao et al., *FEBS Letters*, 351:201–206 (1994); Johnston et al., *Nature*, 370:151–153 (1994); Miyazaki et al., *Science*, 266:1045–1047 (1994); Russell et al., *Science*, 366:1042–1044 (1994); Witthuhn et al., *Nature*, 370:153–157 (1994)). However, as discussed above, a Janus kinase or cytokine receptor family subunit which is not naturally found or used in a given cell may be of particular utility as a PSD, in that such a molecule may either have greater kinase activity and thus be more efficient at promoting cell growth, or it may have less constitutive activity and thus be more readily modulated by clustering.

In yet another preferred embodiment, the present invention relates to T cells containing single chimeric polypeptide receptors that drive both proliferation and effector function through the same inducer molecule. Thus, the extracellular inducer-responsive clustering domain is linked via a transmembrane domain to two signal transducing domains in tandem. One signal transducing domain contains the proliferation signal (as described above) while the other signal transducing domain contains an effector function signal. In a particularly preferred embodiment, the effector signaling domain from a member of the Syk tyrosine kinase family which activates cytolysis, Syk or ZAP-70, is in a chimeric receptor with a proliferation signaling domain from a Janus kinase, JAK1, JAK2, JAK3, Tyk2 or Ptk-2.

In another particularly preferred embodiment, the effector function signaling domain from ζ, η, the FcεR1-β and -γ chains, MB1 (Igα) and B29 (Igβ), BLV gp30, or the CD3γ, δ and ε chains, which also activates cytolysis, is in a chimeric receptor with a proliferation signaling domain from a Janus kinase, JAK1, JAK2, JAK3, Tyk2 or Ptk-2 or a cytokine receptor subunit. These hybrid receptors are contemplated to induce not only antigen-specific proliferation, but the activation of antigen-specific cytotoxic or helper effector function activity as well.

In yet another preferred embodiment, the present invention relates to engineered T cells expressing CPRs which already contain a chimeric effector function receptors. These dual chimera receptor-expressing T cells respond to specific antigen by activating cytolytic or helper effector function, and may respond to the same or a different antigen by proliferating as well. It is thus desirable to engineer a T cell so that it can become activated to proliferate at the disease site, as well as to kill its target, in a manner dependent only upon the presence of the appropriate antigen-expressing cell. In this preferred embodiment, the two chimeric receptors are provided to the cell as separate molecules. As an example, chimeric proliferation receptors which contain an ECD which recognizes HIV antigens are introduced into cytotoxic T cells expressing a chimeric effector function receptor which contains an ECD which recognizes the same or different HIV antigens. This will allow both the proliferation of and cytotoxic actions of the engineered cells upon contact with HIV infected cells, even in the absence of IL-2.

The chimeric construct, which encodes the chimeric protein according to this invention will be prepared in conventional ways. Since, for the most part, natural sequences may be employed, the natural genes may be isolated and manipulated, as appropriate, so as to allow for the proper joining of the various domains. Thus, one may prepare the truncated portion of the sequence by employing the polymerase chain reaction (PCR), using appropriate primers which result in deletion of the undesired portions of the gene. Alternatively, one may use primer repair, where the sequence of interest may be cloned in an appropriate host. In either case, primers may be employed which result in termini, which allow for annealing of the sequences to result in the desired open reading frame encoding the chimeric protein. Thus, the sequences may be selected to provide for restriction sites which are blunt-ended, or have complementary overlaps.

If desired, the extracellular domain may also include the transcriptional initiation region, which will allow for expression in the target host. Alternatively, one may wish to provide for a different transcriptional initiation region, which may allow for constitutive or inducible expression, depending upon the target host, the purpose for the introduction of the subject chimeric protein into such host, the level of expression desired, the nature of the target host, and the like. Thus, one may provide for expression upon differentiation or maturation of the target host, activation of the target host, or the like.

A wide variety of promoters have been described in the literature, which are constitutive or inducible, where induction may be associated with a specific cell type or a specific level of expression. Alternatively, a number of viral promoters are known which may also find use. Promoters of interest include the β-actin promoter, SV40 early and late promoters, immunoglobulin promoter, human cytomegalovirus promoter, and the Friend spleen focus-forming virus promoter. The promoters may or may not be associated with enhancers, where the enhancers may be naturally associated with the particular promoter or associated with a different promoter.

The sequence of the open reading frame may be obtained from genomic DNA, cDNA, or be synthesized, or combinations thereof. Depending upon the size of the genomic DNA and the number of introns, one may wish to use cDNA or a combination thereof. In many instances, it is found that introns stabilize the mRNA. Also, one may provide for non-coding regions which stabilize the mRNA.

A termination region will be provided 3' to the cytoplasmic domain, where the termination region may be naturally

associated with the cytoplasmic domain or may be derived from a different source. For the most part, the termination regions are not critical and a wide variety of termination regions may be employed without adversely affecting expression.

The various manipulations may be carried out in vitro or may be introduced into vectors for cloning in an appropriate host, e.g., *E. coli*. Thus, after each manipulation, the resulting construct from joining of the DNA sequences may be cloned into an expression vector. The sequence may be screened by restriction analysis, sequencing, or the like to insure that it encodes the desired chimeric protein.

The chimeric construct may be introduced into the target cell in any convenient manner. Techniques include calcium phosphate or DEAE-dextran mediated DNA transfection, electroporation, protoplast fusion, liposome fusion, biolistics using DNA-coated particles, and infection, where the chimeric construct is introduced into an appropriate virus (eg retrovirus, adenovirus, adeno-associated virus, Herpes virus, Sindbis virus, papilloma virus), particularly a non-replicative form of the virus, or the like. In addition, direct injection of naked DNA or protein- or lipid-complexed DNA may also be used to introduce DNA into cells.

Once the target host has been transformed, integration will usually result. However, by appropriate choice of vectors, one may provide for episomal maintenance. A large number of vectors are known which are based on viruses, where the copy number of the virus maintained in the cell is low enough to maintain the viability of the cell. Illustrative vectors include SV40, EBV and BPV.

It is also contemplated that the introduction of the chimeric constructs of the present invention into cells may result in the transient expression of the CPRs. Such transient expression may be preferable if a short-term therapeutic effect is desired. Unstable replication or the absence of DNA replication may result, for example, from adenovirus infection or transformation with naked DNA.

Once one has established that the transformed host cell expresses the CPR of the present invention in accordance with the desired regulation and at a desired level, one may then determine whether the CPR is functional in the host cell in providing for the desired proliferation signal. One may use established methodology for measuring proliferation to verify the functional capability of the CPR. The proliferative response of cells can be measured by a variety of techniques known to those skilled in the art. For example, DNA synthesis can be measured by the incorporation of either tritiated thymidine or orotic acid. The incorporation of bromodeoxyuridine into newly synthesized DNA can be measured by immunological staining and the detection of dyes, or by ELISA (Enzyme-linked immunosorbent assay) (Doyle et al., *Cell and Tissue Culture: Laboratory Procedures*, Wiley, Chichester, England, (1994)). The mitotic index of cells can be determined by staining and microscopy, by the fraction labeled mitoses method or by FACS analysis (Doyle et al., supra, (1994); Dean, *Cell Tissue Kinet.* 13:299–308 (1980); Dean, *Cell Tissue Kinet.* 13:672–681 (1980)). The increase in cell size which accompanies progress through the cell cycle can be measure by centrifugal elutriation (Faha et al., *J Virol.* 67:2456–2465 (1993)). Increases in the number of cells may also be measured by counting the cells, with or without the addition of vital dyes. In addition, signal transduction can also be measured by the detection of phosphotyrosine, the in vitro activity of tyrosine kinases from activated cells, c-myc induction, and calcium mobilization as described in the Examples infra.

As described previously in the specific embodiments, the subject CPRs may be used to direct the proliferation of immune cells with effector function. The CPRs may be introduced into cells that already contain a chimeric receptor construct that stimulates effector function upon contact with a target inducer. The two chimeric constructs may respond to the same or different inducers. Alternatively, a hybrid CPR may be used which contains both a proliferation signaling domain and an effector function signaling domain. These cells would respond to a single target inducer by proliferating and by expressing effector function. Thus, these lymphocytes can be activated by any group of cells which contain specific membrane proteins or antigens which may be distinguished from the membrane proteins or antigens on normal cells. For example, neoplastic cells, virus-infected cells, parasiteinfected cells, or any other diseased cells would be targets for CEPR-containing lymphocytes.

Among the lymphocytes which can be used to treat human disease are cytotoxic CD8+ T cells (CTLs) which have been engineered with CEPRs containing ECDs which recognize specific antigens and can be used to kill infected cells in a variety of viral, and parasitic diseases, where the infected cells express the antigens from the pathogen. In particular, CEPR-CTLs would be particularly effective against viral diseases where transplanted autologous CTLs have shown some efficacy, such as CMV (Reusser et al, *Blood*, 78:1373–1380 (1991), Riddell et al., *Science*, 257:238–241 (1992)) or where explanted and expanded CTLs continued to have cytolytic activity against virally infected cells, such as HIV (Lieberman et al, *Aids Res. and Human Retroviruses*, 11:257–271 (1995)). These CEPRs can be constructed with ECDs which recognize the viral envelope proteins. For example, SAbs which recognize either gp120 or gp41, or the CD4 extracellular domain which recognizes gp120 can be used to engineer HIV-specific CTLs. CEPR-CTLs can also be engineered for use against other viruses, such as Hepatitis B virus, Hepatitis C virus, Kaposi's sarcoma associated Herpes virus, the Herpes Simplex viruses, Herpes Zoster virus, and papilloma viruses. Another target for the engineered CTLs are neoplastic cells which express cancer-specific neoantigens or over-express specific membrane proteins. Examples include the IL-14 receptor, CD19 and CD20 for B cell lymphoma, the Lewis Y and CEA antigens for a variety of carcinomas, the Tag72 antigen for breast and colorectal cancer, EGF-R for lung cancer, and the HER-2 protein which is often amplified in human breast and ovarian carcinomas. As an example, human Heregulin (Hrg), a protein similar in structure to Epidermal Growth Factor (EGF), has been identified as a ligand for the HER-2 protein (Holmes et al., *Science* (1992) 256:1205–1210). The extracellular domain of Hrg could be used as an ECD to form a chimeric construct of the present invention to direct T cells to kill breast carcinoma cells. CEPR-CTLs can also be used to target autoimmune cells in the treatment of autoimmune diseases such as Systemic Lupus Erythematosis (SLE), myasthenia gravis, diabetes, rheumatoid arthritis, and Grave's disease.

$CD4^+$ helper T cells (THs) engineered with CEPRs containing ECDs which recognize specific antigens can also be used to treat human disease. In particular, lymphokine production by CEPR-THs may be effective against cancer cells and mycobacterial infections, including *Mycobacterium avium*, *Mycobacterium tuberculosis* and *Mycobactium leprae*.

Chimeric proliferation receptors which do not contain effector function signaling domains may also be of use in the treatment of human disease. Various cell types containing

the CPR constructs described above may be grown in an appropriate nutrient medium for expansion or may be expanded directly in the body via signaling through the CPR, depending on the cell type, and used in a variety of ways. For example, the expanded cells may be used to reconstruct existing tissue or provide new tissue in transplantation therapy. In a particular example, keratinocytes, used for replacement of skin in the case of burns, may be grown to form a continuous layer prior to application. Alternatively, the keratinocytes may be used in the case of plastic surgery to replace skin removed from the host for use at another site.

Other cell types that would be of particular interest for expansion after delivery of the CPRs of the subject invention are islets of Langerhans which may be grown and introduced into a host by capsules or other means, for the production of insulin. Retinal epithelial cells may also be expanded and injected or implanted into the subretinal space of the eye to treat visual disorders, such as macular degeneration. Immune cells, described in detail above, may be expanded ex vivo and injected into the bloodstream or elsewhere to treat immune deficiency. Myoblasts may be expanded with the present invention and injected at various sites to treat muscle wasting diseases such as Duchenne muscular dystrophy. Hepatocytes may be expanded for use in liver regeneration. Endothelial cells may also be expanded to repair blood vessels or to deliver proteins to the circulation. Nerve cells which ordinarily do not proliferate may be targets for expression by using the CPRs of present invention. In addition cells which will not proliferate in vitro, and therefore cannot be manipulated or genetically engineered may be ideal recipients of the CPRs of the present invention.

Additional types of cells that would benefit from the subject CPR constructs include cells that have genes previously introduced or simultaneously introduced with a CPR which may serve in protein production or to correct a genetic defect. Production of proteins may include growth factors, such as, erythropoietin, G-CSF, M-CSF, and GM-CSF, epidermal growth factor, platelet derived growth factor, human growth factor, transforming growth factor, etc; lymphokines, such as the interleukins; hormones, such as ACTH, somatomedin, insulin, angiotensin, etc.; coagulation factors, such as Factor VIIIC; deoxyribonuclease for treating cystic fibrosis; glucocerebrosidase for treating Gaucher's disease; normal versions of proteins associated with genetic diseases such as adenosine deaminase or the CFTR protein associated with cystic fibrosis; protective agents, such as α1-antitrypsin; regulatory proteins or enzymes associated with the production of amino acid free products, such as the expression of tyrosine hydroxylase for the production of L-dopamine, and the like.

The recipient of genetically modified allogeneic cells can be immunosuppressed to prevent the rejection of the transplanted cells. In the case of immunocompromised patients, no pretransplant therapy may be required. Another alternative source of cells to be transplanted are so-called "universal donor" cells which have been genetically engineered so that they do not express antigens of the major histocompatibility complex or molecules which function in antigen presentation.

High-titer retroviral producer lines are used to transduce the chimeric proliferation receptor constructs into autologous or allogeneic human T-cells, hematopoietic stem cells or other cells, described above through the process of retroviral mediated gene transfer as described by Lusky et al. in (1992) *Blood* 80:396. In addition to the gene encoding the chimeric proliferation receptor, additional genes may be included in the retroviral construct. These include genes such as the thymidine kinase or cytosine deaminase genes (Borrelli et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:7572) which acts as a suicide gene for the marked cells if the patient is exposed to gancyclovir or 5'-fluorouracil (5FU), respectively. Thus, if the percentage of marked cells is too high, gancyclovir or 5FU may be administered to reduce the percentage of cells expressing the chimeric receptors. In addition, if the percentage of marked cells needs to be increased, the multi-drug resistance gene can be included (Sorrentino et al. (1992) *Science* 257:99) which functions as a preferential survival gene for the marked cells in the patients if the patient is administered a dose of a chemotherapeutic agent such as taxol. Therefore, the percentage of marked cells in the patients can be titrated to obtain the maximum therapeutic benefit.

In addition, high-titer adenoviral producer lines may be used to transduce the chimeric proliferation receptor constructs into autologous or allogeneic nerve cells, hematopoietic cells including stem cells, islets of Langerhans, keratinocytes, muscle cells or other cells following the methods of adenoviral mediated gene transfer as described by Finer et al. in *Blood*, 83:43–50 (1994). Similar to the procedure described above, other genes may be included in the adenoviral constructs in addition to the chimeric proliferation receptor in the recipient cell. After introduction of the construct into the cell type of interest, the cells may be expanded in an appropriate medium well know in the art and used in a variety of ways previously described.

The following examples are by way of illustration and not by way of limitation.

EXPERIMENTAL

EXAMPLE 1

Construction of CPRs comprising a ligand-receptor (CD4) extracellular clustering domain and a Janus kinase or cytokine receptor subunit proliferation signaling domain.

Expression vectors for CD4-Janus kinase and CD4-cytokine receptor subunit hybrids were created using pIK1.1F3Sal. This plasmid was made by introducing a SalI site into pIK1.1F3 (U.S. Pat. No. 5,359,046) which directs the expression CD4-ζ, a chimeric protein comprised of the human CD4 extracellular (EXT) and transmembrane (TM) domains (residues 1 to 395 of mature CD4) fused to the cytoplasmic (CYT) domain of human ζ. The SalI site was introduced by oligonucleotide-directed mutagenesis using single stranded pIK1.1F3 DNA with oligo 1 as the primer. pIK1.1F3Sal was identified by restriction analysis and its sequence confirmed by Sanger dideoxynucleotide sequencing. The creation of the SalI site results in the insertion of an Asp codon at the junction of CD4 TM and ζ CYT, and permits the replacement of ζ CYT domain with a Janus kinases or cytokine receptor subunit CYT domain with the retention of a single Asp residue at the junction. Derivatives lacking the extra Asp codon or containing other oligo- or polypeptide linkers are constructed by oligonucleotide-directed mutagenesis (Zoller and Smith, (1982) *Nucleic Acids Res*,. 10:6487–6500). In each example below, the correct expression plasmid was identified by restriction mapping and its structure confirmed by DNA sequencing.

a) Construction of CD4-mJAK1

pIKCD4-mJAK1 directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1 to 395) joined at their C-terminus to the entire mouse JAK1 Janus kinase by an Asp residue. This plasmid was constructed from three DNA fragments: 1) a vector fragment of 5.7 kb obtained by digestion of pIK1.1F3Sal with SalI and

ApaI, 2) a 2.6 kb fragment encoding the N-terminus of mJAK1 obtained by digestion of pBluescriptKSmJAK1 (provided by James Ihle & Bruce Witthuhn, St Jude Children's Research Hospital, Memphis, Tenn.) with NcoI and SstI, and ligation to a SalI-NcoI adaptor consisting of oligonucleotides 2 & 3 (SEQ ID NO: 2 & 3), and 3) a 0.9 kb fragment encoding the C-terminus of mJAK1 obtained by digestion of pBluescriptKSmJAK1 with SstI and NdeI, and ligation to an NdeI-ApaI adaptor consisting of oligonucleotides 4 & 5 (SEQ ID NO: 4 & 5).

b) Construction of CD4-mJAK2

pIKCD4-mJAK2 directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1–395) joined at their C-terminus to the entire mouse JAK2 Janus kinase by an Asp residue. This plasmid was constructed in two steps. First, an intermediate plasmid was constructed from two DNA fragments: 1) a vector fragment of 5.7 kb obtained by digestion of pIK1.1F3Sal with SalI and ApaI and modification of the cohesive ends with T4 polymerase and dNTPs to create blunt ends, and 2) a 3.7 kb fragment encoding the entire mJAK2 protein obtained by digestion of pBluescriptSKmJAK2 (provided by James Ihle & Bruce Witthuhn, St Jude Children's Research Hospital, Memphis, Tenn.) with NotI and NheI and extension of the cohesive ends with T4 polymerase and dNTPs to create blunt ends. A clone with the insert in the correct orientation, having the blunted SalI and NotI sites joined, was identified and used to prepare a single-stranded DNA template. Secondly, this template was used for oligonucleotide-directed mutagenesis with oligonucleotide 6 (SEQ ID NO:6) as a primer to fuse amino acid 1 of mJAK2 in-frame to the Asp residue following the CD4 TM region. The correct expression plasmid was identified by colony hybridization using oligonucleotide 7 (SEQ ID NO:7) as a probe.

c) Construction of CD4-mJAK3

pIKCD4-mJAK3 directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1–395) joined at their C-terminus to the entire mouse JAK2 Janus kinase by an Asp residue. This plasmid was constructed from three DNA fragments: 1) a vector fragment of 5.7 kb obtained by digestion of pIK1.1F3Sal with SalI and ApaI, 2) a 1.3 kb fragment encoding the mJAK3 N-terminus obtained by digestion of pBluescriptSKmJAK3 (provided by James Ihle & Bruce Witthuhn, St Jude Children's Research Hospital, Memphis, Tenn.) with Eco47III and EcoRI, and ligation to a SalI-Eco47III adaptor consisting of oligonucleotides 8 & 9 (SEQ ID NO:8 & 9), and 3) a 2.2 kb fragment encoding the mJAK3 C-terminus obtained by digestion of pBluescriptSKmJAK3 with EcoRI and BamHI, and ligation to a BamHI-ApaI adaptor consisting of oligonucleotides 10 & 11 (SEQ ID NO:10 & 11).

d) Construction of CD4-hTyk2

pIKCD4-hTyk2 directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1–395) joined at their C-terminus to the entire human Tyk2 Janus kinase by an Asp residue. This plasmid was constructed in two steps. First, an intermediate plasmid was constructed from three DNA fragments: 1) a vector fragment of 5.7 kb obtained by digestion of pIK1.1F3Sal with SalI, extension of the cohesive end with T4 polymerase and dNTPs to create a blunt end, followed by digestion with ApaI, and 2) a 1.1 kb fragment encoding the N-terminus of hTyk2 obtained by digestion of pRCFwt (provided by Sandra Pellegrini, Institut Pasteur, Paris) with SphI, extension of the cohesive end with T4 polymerase and dNTPs, followed by digestion with SacII, and 3) a 2.6 kb fragment encoding the C-terminus of hTyk2 obtained by digestion of pRCFwt with SacII and ApaI. Secondly, a single-stranded DNA template was prepared from this intermediate plasmid and used for oligonucleotide-directed mutagenesis with oligonucleotide 12 (SEQ ID NO:12) as a primer to fuse amino acid 1 of hTyk2 in-frame to the Asp residue following the CD4 coding region. The correct expression plasmid was identified by colony hybridization using oligonucleotide 13 (SEQ ID NO:13)as probe.

e) Construction of CD4-hJAK3

pIKCD4-hJAK3 directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1–395) joined at their C-terminus to the entire human Tyk2 Janus kinase by an Asp residue. This plasmid was constructed in two steps. First, an intermediate plasmid was constructed from three DNA fragments: 1) a vector fragment of 5.7 kb obtained by digestion of pIK1.1F3Sal with SalI and ApaI, and extension of the cohesive ends with T4 polymerase and dNTPs to create blunt ends, and 2) a 3.6 kb fragment encoding the entire hJAK3 protein obtained by digestion of pBluescriptSKhJAK3 (provided by John O'Shea, National Cancer Institute, Frederick, Md.) with EcoRI and NdeI and extension of the cohesive ends with T4 polymerase and dNTPs to create blunt ends. A clone with the insert in the correct orientation, having the blunted SalI and EcoRI sites joined, was identified and used to prepare a single-stranded DNA template. Secondly, this template was used for oligonucleotide-directed mutagenesis with oligonucleotide 14 (SEQ ID NO:14)as a primer to fuse amino acid 1 of hJAK3 in-frame to the Asp residue following the CD4 TM region. The correct expression plasmid was identified by colony hybridization using oligonucleotide 15 (SEQ ID NO:15)as a probe.

f) Construction of CD4-hIL2Rβ

pIKCD4-hIL2Rβ directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1–395) joined at their C-terminus to the CYT domain of the human IL-2 receptor β subunit (residues 240–525 of the mature polypeptide) by an Asp residue. This plasmid was constructed from two DNA fragments: 1) a vector fragment of 5.7 kb obtained by digestion of pIK1.1F3Sal with ApaI, extension of the cohesive end with T4 polymerase and dNTPs to create a blunt end, followed by digestion with SalI, and 2) a 0.9 kb fragment encoding the hIL-2Rβ CYT domain obtained by digestion of a PCR-generated DNA fragment with SalI and EcoRV. The PCR-generated fragment was obtained by 1) isolating mRNA from normal human CD8-positive T cells with a FastTrack kit (Invitrogen, San Diego, Calif.), 2) using the mRNA to prepare single-stranded cDNA using a cDNA Cycle kit (Invitrogen, San Diego, Calif.) with oligonucleotide 16 (SEQ ID NO:16) as a primer, and 3) amplifying the single-stranded cDNA by PCR using oligonucleotides 17 & 18 (SEQ ID NO:17 & 18) as primers to generate a fragment which incorporates SalI and EcoRV sites at the 5' and 3' ends, respectively.

g) Construction of CD4-IL2Rγ

pIKCD4-IL2Rγ directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1–395) joined at their C-terminus to the CYT domain of the human IL-2 receptor γ subunit (residues 262–347 of the mature polypeptide) by an Asp residue. This plasmid was constructed from two DNA fragments: 1) a vector fragment of 5.7 kb obtained by digestion of pIK1.1F3Sal with ApaI, extension of the cohesive end with T4 polymerase and dNTPs to create a blunt end, followed by digestion with SalI, and 2) a 0.3 kb fragment encoding the hIL-2Rγ CYT domain obtained by digestion of a PCR-generated DNA fragment with SalI and EcoRV. The PCR-generated fragment was

obtained by 1) isolating a hIL-2Rγ DNA clone from a λ cDNA library made from activated human T cells (Clontech, Palo Alto, Calif.) using oligonucleotides 19 & 20 (SEQ ID NO:19 & 20)as probes, 2) subcloning an EcoRI fragment containing the hIL-2Rγ CYT domain (residues 268–347), 3) using the subclone DNA to carry out PCR with oligos 21 and 22 as primers to generate a fragment in which the codons for hIL-2Rγ residues 262–267 were recreated, the EcoRI site was removed, and in which SalI and EcoRV sites were incorporated at the 5' and 3' ends, respectively.

EXAMPLE 2

CPRs containing an antibody extracellular clustering domain and a Janus kinase or cytokine receptor subunit proliferation signaling domain.

Expression vectors for SAb-Janus kinase and SAb-cytokine receptor subunit hybrids are created by replacing the CD4 EXT domain in CD4-Janus kinase, and CD4-cytokine receptor subunit hybrids (examples 1*a* to 1*g*) with the EXT domain of F15γ2, a single-chain antibody-ζ chimeric receptor, contained in plasmid pRT43.2F15γ2. F15γ2 is comprised of (from N- to C-terminus) of: 1) the signal sequence and V_K domain of human anti-HIV gp41 MAb 98.6 (residues 1–107 of the mature protein), 2) a 14 amino acid peptide linker (Gly-Ser-Thr-Ser-Gly-Ser-Gly-Lys-Ser-Ser-Glu-Gly-Lys-Gly), 3) the V_H domain of MAb 98.6 (residues 1–113 of the mature protein), 4) the hinge, CH2 and CH3 domains of the human IgG2 heavy chain constant region (residues 226 to 477), 5) the 18 residue human IgG3 M1 membrane hinge, 6) the CD4 TM domain (residues 372–395), and 7) the ζ CYT domain (residues 31–142). The presence of the IgG2 heavy chain constant domain allows such SAb-Janus kinase and SAb-cytokine receptor subunit constructs to form disulfide-linked dimers. Derivatives which lack the constant domain, and thus do not dimerize, are made by oligonucleotide directed mutagenesis. Other derivatives lacking the Asp codon or containing other oligo- or polypeptide linkers at the junction of CD4 TM and the CYT domain of the Janus kinase or cytokine receptor subunit are constructed by oligonucleotide directed mutagenesis. In each example, the correct expression plasmid is identified by restriction mapping and its structure confirmed by DNA sequencing.

a) Construction of SAb-mJAK1

pIKSAb-mJAK1 directs the expression of a hybrid protein consisting of the SAb EXT and CD4 TM domains of F15γ2 joined at their C-terminus to the entire mouse JAK1 Janus kinase by an Asp residue. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 4.3 kb obtained by digestion of the expression plasmid pIK1.1 with EcoRI and ApaI, 2) a fragment of 1.6 kb encoding the SAb EXT domain and part of the CD4 TM domain, obtained by digestion of pRT43.2F15γ2 with EcoRI and NgoMI, and 3) a 3.7 kb fragment encoding the remainder of the CD4 TM domain and the entire mJAK1 protein, obtained by digestion of pIKCD4-mJAK1 with NgoMI and ApaI.

b) Construction of SAb-mJAK2

pIKSAb-mJAK2 directs the expression of a hybrid protein consisting of the SAb EXT and CD4 TM domains of F15γ2 joined at their C-terminus to the entire mouse JAK2 Janus kinase by an Asp residue. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.6 kb encoding the entire mJAK2 protein, obtained by digestion of pIKCD4-mJAK2 with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-mJAK1 with SphI and BamHI, and 3) a fragment of 1.0 kb encoding the remainder of the SAb EXT domain and the CD4 TM domain, obtained by digestion of pIKSAb-mJAK1 with BamHI and SalI.

c) Construction of SAb-mJAK3

pIKSAb-mJAK3 directs the expression of a hybrid protein consisting of the SAb EXT and CD4 TM domains of F15γ2 joined at their C-terminus to the entire mouse JAK2 Janus kinase by an Asp residue. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire mJAK3 protein, obtained by digestion of pIKCD4-mJAK3 with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-mJAK1 with SphI and BamHI, and 3) a fragment of 1.0 kb encoding the remainder of the SAb EXT domain and the CD4 TM domain, obtained by digestion of DIKSAb-mJAK1 with BamHI and SalI.

d) Construction of SAb-hTyk2

pIKSAb-hTyk2 directs the expression of a hybrid protein consisting of the SAb EXT and CD4 TM domains of F15γ2 joined at their C-terminus to the entire human Tyk2 Janus kinase by an Asp residue. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.5 kb encoding the C-terminus of hTyk2, obtained by digestion of pIKCD4-hTyk2 with EcoRI and BspEI, 2) a fragment of 1.6 kb encoding the SAb EXT domain and a portion of the CD4 TM domain, obtained by digestion of pRT43.2F15γ2 with EcoRI and NgoMI, and 3) a fragment of 0.4 kb encoding the remainder of the CD4 TM domain and the N-terminus of the hTyk2 protein, obtained by digestion of pIKCD4-hTyk2 with NgoMi and BspEI.

e) Construction of Sab-CD4-hJAK3

pIKSAb-hJAK3 directs the expression of a hybrid protein consisting of the SAb EXT and CD4 TM domains of F15γ2 joined at their C-terminus to the entire human JAK3 Janus kinase by an Asp residue. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire mJAK3 protein, obtained by digestion of pIKCD4-hJAK3 with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-mJAK1 with SphI and BamHI, and 3) a fragment of 1.0 kb encoding the remainder of the SAb EXT domain and the CD4 TM domain, obtained by digestion of pIKSAb-mJAK1 with BamHI and SalI.

f) Construction of SAb-IL2Rβ

pIKSAb-hIL2Rβ directs the expression of a hybrid protein consisting of the SAb EXT and CD4 TM domains of F15γ2 joined at their C-terminus to the human IL2Rβ CYT domain by an Asp residue. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 5.0 kb encoding the IL-2Rβ CYT domain, obtained by digestion of pIKCD4-hIL2Rβ with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-mJAK1 with SphI and BamHI, and 3) a fragment of 1.0 kb encoding the remainder of the SAb EXT domain and the CD4 TM domain, obtained by digestion of pIKSAb-mJAK1 with BamHI and SalI.

g) Construction of SAb-IL2Rγ

pIKSAb-hIL2Rγ directs the expression of a hybrid protein consisting of the SAb EXT and CD4 TM domains of F15γ2 joined at their C-terminus to the human IL2Rγ CYT domain by an Asp residue. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 4.4 kb encoding the IL-2Rγ CYT domain, obtained by digestion of pIKCD4-hIL2Rγ with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-mJAK1 with SphI and BamHI, and 3) a fragment of 1.0 kb encoding the remainder of the SAb EXT domain and the CD4 TM domain, obtained by digestion of pIKSAb-mJAK1 with BamHI and SalI.

EXAMPLE 3

CPRs comprising a ligand-receptor (CD4) extracellular clustering domain, a ζ family signalling domain and a Janus kinase or cytokine receptor subunit proliferation signaling domain.

This class of chimeric receptors were created by the insertion of a ζ family CYT signaling domain (e.g. ζ, η, the FcRε γ subunit, B29, and CD3 γ, δ and ε subunits) into a CPR between the TM domain and proliferation signaling (Janus kinase or cytokine receptor subunit) domain. These chimeric receptors were constructed from pIK1.1F3SalB, an intermediate plasmid based on pIK1.1F3 (which encodes CD4-ζ). A SalI site was introduced into the CD4-ζ coding sequence between the last amino acid and stop codon by oligonucleotide-directed mutagenesis using pIK1.1F3 single-stranded DNA with oligonucleotide 23 (SEQ ID NO:23) as a primer and oligonucleotide 24 (SEQ ID NO:24) to identify the correct clone by colony hybridization. This results in the addition of 2 residues (Val-Asp) at the carboxyl terminus of CD4-ζ. The proliferation signaling domain of a Janus kinase or cytokine receptor subunit was then joined at the C-terminus of CD4-ζ using the unique SalI site which adds a Val-Asp dipeptide at the junction. Derivatives lacking the Val-Asp dipeptide or containing other oligo- or polypeptide linkers are constructed by oligonucleotide-directed mutagenesis. A similar strategy is used to create CPRs containing a ζ family signaling domain at the C-terminus of the chimeric protein (e.g., CD4-Janus kinase-ζ and CD4-cytokine receptor subunit-ζ) by inserting the ζ family CYT domain after the proliferation signalling CYT domain.

a) Construction of CD4-ζ-mJAK1

pIKCD4-ζ-mJAK1 directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1 to 395) and ζ CYT domain joined at their C-terminus to the entire mouse JAK1 Janus kinase by a Val-Asp dipeptide. This plasmid was constructed from two DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire mJAK1 protein, obtained by digestion of pIKCD4-mJAK1 with SphI and SalI, 2) a 1.8 kb fragment encoding the CD4 EXT and TM domains and the ζ CYT domain, obtained by digestion of pIK1.1F3SalB with SphI and SalI.

b) Construction of CD4-ζ-mJAK2

pIKCD4-ζ-mJAK2 directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1 to 395) and ζ CYT domain joined at their C-terminus to the entire mouse JAK2 Janus kinase by a Val-Asp dipeptide. This plasmid was constructed from two DNA fragments: 1) a vector fragment of 7.6 kb encoding the entire mJAK2 protein, obtained by digestion of pIKCD4-mJAK2 with SphI and SalI, 2) a 1.8 kb fragment encoding the CD4 EXT and TM domains and the ζ CYT domain, obtained by digestion of pIK1.1F3SalB with SphI and SalI.

c) Construction of CD4-ζ-mJAK3

pIKCD4-ζ-mJAK3 directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1 to 395) and ζ CYT domain joined at their C-terminus to the entire mouse JAK3 Janus kinase by a Val-Asp dipeptide. This plasmid was constructed from two DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire mJAK3 protein, obtained by digestion of pIKCD4-mJAK3 with SphI and SalI, 2) a 1.8 kb fragment encoding the CD4 EXT and TM domains and the ζ CYT domain, obtained by digestion of pIK1.1F3SalB with SphI and SalI.

d) Construction of CD4-ζ-hTyk2

pIKCD4-ζ-hTyk2 directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1 to 395) and ζ CYT domain joined at their C-terminus to the entire human Tyk2 Janus kinase by a Val-Asp dipeptide. This plasmid was constructed from three DNA fragments: 1) a vector fragment of 7.5 kb encoding the C-terminus of hTyk2, obtained by digestion of pIKCD4-hTyk2 with EcoRI and BspEI, 2) a 1.7 kb fragment encoding the CD4 EXT and TM domains and the ζ CYT domain, obtained by digestion of pIK1.1F3SalB with EcoRI and SalI, and 3) a 0.3 kb fragment encoding the N-terminus of hTyk2, obtained by digestion of pIK1.1F3SalB with SalI and BspEI.

e) Construction of CD4-ζ-hJAK3

pIKCD4-ζ-hJAK3 directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1 to 395) and ζ CYT domain joined at their C-terminus to the entire human JAK3 Janus kinase by a Val-Asp dipeptide. This plasmid was constructed from two DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire hJAK3 protein, obtained by digestion of pIKCD4-hJAK3 with SphI and SalI, 2) a 1.8 kb fragment encoding the CD4 EXT and TM domains and the ζ CYT domain, obtained by digestion of pIK1.1F3SalB with SphI and SalI.

f) Construction of CD4-ζ-hIL2Rβ

pIKCD4-ζ-hIL2Rβ directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1 to 395) and ζ CYT domain joined at their C-terminus to the human IL2Rβ CYT domain subunit by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 5.0 kb encoding the hIL2Rβ CYT domain, obtained by digestion of pIKCD4-hIL2Rβ with SphI and SalI, 2) a 1.8 kb fragment encoding the CD4 EXT and TM domains and the ζ CYT domain, obtained by digestion of pIK1.1F3SalB with SphI and SalI.

g) Construction of CD4-ζ-hIL2Rγ

pIKCD4-ζ-hIL2Rγ directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1 to 395) and ζ CYT domain joined at their C-terminus to the human IL2Rγ CYT domain by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 4.4 kb encoding the hIL2Rγ CYT domain, obtained by digestion of pIKCD4-hIL2Rβ with SphI and SalI, 2) a 1.8 kb fragment encoding the CD4 EXT and TM domains and the ζ CYT domain, obtained by digestion of pIK1.1F3SalB with SphI and SalI.

EXAMPLE 4

CPRs containing an antibody extracellular clustering domain, a ζ family signaling domain and a Janus kinase or cytokine receptor subunit proliferation signaling domain.

This class of chimeric receptors are created by the insertion of a ζ family CYT signaling domain (e.g. ζ, η, the FcRε γ subunit, B29, and CD3 γ, δ and ε subunits) into an antibody-based CPR between the TM domain and proliferation signaling (Janus kinase or cytokine receptor subunit) domain. These chimeric receptors are constructed from CD4-ζ-Janus kinase and CD4-ζ-cytokine receptor subunit CPRs, by substituting an antibody-based EXT clustering domain for the CD4 EXT domain. The proliferation signalling domain of a Janus kinase or cytokine receptor subunit is joined at the C-terminus of SAb-ζ by a Val-Asp dipeptide. Derivatives lacking the Val-Asp dipeptide or containing other oligo- or polypeptide linkers are constructed by oligonucleotide-directed mutagenesis. A similar strategy is used to create CPRs containing a ζ family signaling domain at the C-terminus of the chimeric protein (e.g., SAb-Janus kinase-ζ and SAb-cytokine receptor subunit-ζ) by inserting the ζ family CYT domain after the proliferation signalling CYT domain.

a) Construction of SAb-ζ-mJAK1

pIKSAb-ζ-mJAK1 directs the expression of a hybrid protein consisting of the 98.6 SAb EXT, CD4 TM and ζ CYT domain joined at their C-terminus to the entire mouse JAK1 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 4.3 kb obtained by digestion of the expression plasmid

pIK1.1 with EcoRI and ApaI, 2) a fragment of 1.6 kb encoding the SAb EXT domain and part of the CD4 TM domain, obtained by digestion of pRT43.2F15γ2 with EcoRI and NgoMI, and 3) a 4.0 kb fragment encoding the remainder of the CD4 TM domain, the ζ CYT domain and the entire mJAK1 protein, obtained by digestion of pIKCD4-ζ-mJAK1 with NgoMI and ApaI.

b) Construction of SAb-ζ-mJAK2

pIKSAb-ζ-mJAK2 directs the expression of a hybrid protein consisting of the 98.6 SAb EXT, CD4 TM and ζ CYT domain joined at their C-terminus to the entire mouse JAK2 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.6 kb encoding the entire mJAK2 protein, obtained by digestion of pIKCD4-mJAK2 with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-ζ-mJAK1 with SphI and BamHI, and 3) a fragment of 1.4 kb encoding the remainder of the SAb EXT domain, the CD4 TM domain and the ζ CYT domain, obtained by digestion of pIKSAb-ζ-mJAK1 with BamHI and SalI.

c) Construction of SAb-ζ-mJAK3

pIKSAb-ζ-mJAK3 directs the expression of a hybrid protein consisting of the 98.6 SAb EXT, CD4 TM and ζ CYT domain joined at their C-terminus to the entire mouse JAK3 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire mJAK3 protein, obtained by digestion of pIKCD4-mJAK3 with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-ζ-mJAK1 with SphI and BamHI, and 3) a fragment of 1.4 kb encoding the remainder of the SAb EXT domain, the CD4 TM domain and the ζ CYT domain, obtained by digestion of pIKSAb-ζ-mJAK1 with BamHI and SalI.

d) Construction of SAb-ζ-hTyk2

pIKSAb-ζ-hTyk2 directs the expression of a hybrid protein consisting of the 98.6 EXT, CD4 TM and ζ CYT domain joined at their C-terminus to the entire human Tyk2 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.5 kb encoding the C-terminus of hTyk2, obtained by digestion of pIKCD4-hTyk2 with EcoRI and BspEI, 2) a fragment of 1.6 kb encoding the SAb EXT domain and a portion of the CD4 TM domain, obtained by digestion of pIKSAb-ζ-mJAK1 with EcoRI and NgoMI, and 3) a fragment of 1.6 kb encoding the remainder of the CD4 TM domain, the ζ CYT domain and the N-terminus of the hTyk2 protein, obtained by digestion of pIKCD4-ζ-hTyk2 with NgoMI and BspEI.

e) Construction of SAb-ζ-hJAK3

pIKCD4-ζ-hJAK3 directs the expression of a hybrid protein consisting of the 98.6 EXT, CD4 TM and ζ CYT domain joined at their C-terminus to the entire human JAK3 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire hJAK3 protein, obtained by digestion of pIKCD4-hJAK3 with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-ζ-mJAK1 with SphI and BamHI, and 3) a fragment of 1.4 kb encoding the remainder of the SAb EXT domain, the CD4 TM domain and the ζ CYT domain, obtained by digestion of pIKSAb-ζ-mJAK1 with BamHI and SalI.

f) Construction of SAb-ζ-hIL2Rβ

pIKSAb-ζ-hIL2Rβ directs the expression of a hybrid protein consisting of the 98.6 EXT, CD4 TM and ζ CYT domain joined at their C-terminus to the human IL2Rβ CYT domain by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 5.0 kb encoding the hIL2Rβ CYT domain, obtained by digestion of pIKCD4-hIL2Rβ with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-ζ-mJAK1 with SphI and BamHI, and 3) a fragment of 1.4 kb encoding the remainder of the SAb EXT domain, the CD4 TM domain and the ζ CYT domain, obtained by digestion of pIKSAb-ζ-mJAK1 with BamHI and SalI.

g) Construction of SAb-ζ-hIL2Rγ

pIKSAb-ζ-hIL2Rγ directs the expression of a hybrid protein consisting of the 98.6 EXT, CD4 TM and ζ CYT domain joined at their C-terminus to the human IL2Rγ CYT domain by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 4.4 kb encoding the hIL2Rγ CYT domain, obtained by digestion of pIKCD4-hIL2Rγ with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-ζ-mJAK1 with SphI and BamHI, and 3) a fragment of 1.4 kb encoding the remainder of the SAb EXT domain, the CD4 TM domain and the ζ CYT domain, obtained by digestion of pIKSAb-ζ-mJAK1 with BamHI and SalI.

EXAMPLE 5

CPRs containing a ligand-receptor (CD4) extracellular clustering domain, a Syk family kinase signaling domain and a Janus kinase or a cytokine receptor subunit proliferation signaling domain.

This class of chimeric receptors are created by the insertion of a Syk family kinase (e.g., Syk and ZAP-70) into a CPR between the TM domain and proliferation signaling (Janus kinase or cytokine receptor subunit) domain. These chimeric receptors are constructed from CD4-ζ-Janus kinase or CD4-ζ-cytokine receptor subunit CPRs, by replacing the ζ family CYT domain with the entire Syk family polypeptide. CPRs based on the Syk kinase are made from the intermediate plasmid pIK1.1CD4-Syk which directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains joined to the entire human Syk polypeptide by a Glu residue. This plasmid is constructed from two fragments: 1) a vector fragment of 5.7 kb encoding the CD4 EXT and TM domains, obtained by digestion of pIK1.1F3Sal with ApaI, extension of the cohesive end to a blunt end with T4 DNA polymerase and dNTPs, followed by digestion with SalI, and 2) a 1.8 kb PCR fragment encoding human Syk kinase, generated using ψHM3-Syk (provided by Edward Clark, U. of Washington, Seattle, Wash.) as a PCR template with oligonucleotides 25 & 26 (SEQ ID NO:25 & 26) as primers to introduce XhoI and EcoRV sites at the 5' and 3' ends, respectively, followed by digestion with XhoI and EcoRV. The Janus kinase or cytokine receptor subunit is then joined at the C-terminus of CD4-Syk using the unique SalI site which adds a Val-Asp dipeptide at the junction. Derivatives lacking the Val-Asp dipeptide or containing other oligo- or polypeptide linkers are constructed by oligonucleotide-directed mutagenesis. A similar strategy is used to create CPRs containing a Syk family kinase at the C-terminus of the chimeric protein (e.g., CD4-Janus kinase-ζ and CD4-cytokine receptor subunit-ζ) by inserting the Syk family kinase after the proliferation signalling CYT domain.

a) Construction of CD4-Syk-mJAK1

pIKCD4-Syk-mJAK1 directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1 to 395) and the entire Syk protein joined at their C-terminus to the entire mouse JAK1 Janus kinase by a

Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire mJAK1 protein, obtained by digestion of pIKCD4-mJAK1 with SphI and SalI, and 2) a 3.3 kb fragment encoding the CD4 EXT and TM domains and the entire Syk protein, obtained by digestion of pIK1.1CD4-Syk with SphI and SalI.

b) Construction of CD4-Syk-mJAK2

pIKCD4-Syk-mJAK2 directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1 to 395) and the entire Syk protein joined at their C-terminus to the entire mouse JAK2 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 7.6 kb encoding the entire mJAK2 protein, obtained by digestion of pIKCD4-mJAK2 with SphI and SalI, and 2) a 3.3 kb fragment encoding the CD4 EXT and TM domains and the entire Syk protein, obtained by digestion of pIK1.1CD4-Syk with SphI and SalI.

c) Construction of CD4-Syk-mJAK3

pIKCD4-Syk-mJAK3 directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1 to 395) and the entire Syk protein joined at their C-terminus to the entire mouse JAK3 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire mJAK3 protein, obtained by digestion of pIKCD4-mJAK3 with SphI and SalI, and 2) a 3.3 kb fragment encoding the CD4 EXT and TM domains and the entire Syk protein, obtained by digestion of pIK1.1CD4-Syk with SphI and SalI.

d) Construction of CD4-Syk-hTyk2

pIKCD4-Syk-hTyk2 directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1 to 395) and the entire Syk protein joined at their C-terminus to the entire human Tyk2 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.5 kb encoding the C-terminus of hTyk2, obtained by digestion of pIKCD4-hTyk2 with EcoRI and BspEI, 2) a 3.3 kb fragment encoding the CD4 EXT and TM domains and the entire Syk protein, obtained by digestion of pIK1.1CD4-Syk with EcoRI and SalI, and 3) an 0.3 kb fragment encoding the N-terminus of hTyk2, obtained by digestion of pIK1.1F3SalB with SalI and BspEI.

e) Construction of CD4-Syk-hJAK3

pIKCD4-Syk-hJAK3 directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1 to 395) and the entire Syk protein joined at their C-terminus to the entire human JAK3 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire hJAK3 protein, obtained by digestion of pIKCD4-hJAK3 with SphI and SalI, and 2) a 3.3 kb fragment encoding the CD4 EXT and TM domains and the entire Syk protein, obtained by digestion of pIK1.1CD4-Syk with SphI and SalI.

f) Construction of CD4-Syk-hIL2Rβ

pIKCD4-Syk-hIL2Rβ directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1 to 395) and the entire Syk protein joined at their C-terminus to the human IL2Rβ CYT domain by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 5.0 kb encoding the hIL2Rβ CYT domain, obtained by digestion of pIKCD4-hIL2Rβ with SphI and SalI, 2) a 3.3 kb fragment encoding the CD4 EXT and TM domains and the entire Syk protein, obtained by digestion of pIK1.1CD4-Syk with SphI and SalI.

g) Construction of CD4-Syk-hIL2Rγ

pIKCD4-Syk-hIL2Rγ directs the expression of a hybrid protein consisting of the CD4 EXT and TM domains (residues 1 to 395) and the entire Syk protein joined at their C-terminus to the human IL2Rγ CYT domain by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 4.4 kb encoding the hIL2Rβ CYT domain, obtained by digestion of pIKCD4-hIL2Rβ with SphI and SalI, 2) a 3.3 kb fragment encoding the CD4 EXT and TM domains and the entire Syk protein, obtained by digestion of pIK1.1CD4-Syk with SphI and SalI.

EXAMPLE 6

CPRs containing an antibody extracellular clustering domain, and a Syk family kinase signaling domain and Janus kinase & cytokine receptor subunit proliferation signaling domain

This class of chimeric receptors are created by the insertion of a Syk family kinase (e.g. Syk and ZAP-70) into an antibody-based CPR between the TM domain and proliferation signaling (Janus kinase or cytokine receptor subunit) domain. These chimeric receptors are constructed from CD4-Syk-Janus kinase and CD4-Syk-cytokine receptor subunit CPRs, by substituting an antibody-based EXT clustering domain for the CD4 EXT domain. The proliferation signaling domain of a Janus kinase or cytokine receptor subunit is joined at the C-terminus of SAb-Syk by a Val-Asp dipeptide. Derivatives lacking the Val-Asp dipeptide or containing other oligo- or polypeptide linkers are constructed by oligonucleotide-directed mutagenesis. A similar strategy is used to create CPRs containing a Syk family kinase at the C-terminus of the chimeric protein (e.g., SAb-Janus kinase-Syk kinase and SAb-cytokine receptor subunit-Syk kinase) by inserting the Syk family kinase after the proliferation signalling CYT domain.

a) Construction of SAb-Syk-mJAK1

pIKSAb-Syk-mJAK1 directs the expression of a hybrid protein consisting of the 98.6 SAb EXT, CD4 TM and the entire Syk protein joined at their C-terminus to the entire mouse JAK1 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire mJAK1 protein, obtained by digestion of pIKCD4-mJAK1 with SphI and SalI, 2) a fragment of 1.7 kb encoding the SAb EXT domain and part of the CD4 TM domain, obtained by digestion of pIKSAb-mJAK1 with SphI and NgoMI, and 3) a 2.0 kb fragment encoding the remainder of the CD4 TM domain and the entire Syk protein, obtained by digestion of pIKCD4-Syk-mJAK1 with NgoMI and SalI.

b) Construction of SAb-Syk-mJAK2

pIKSAb-Syk-mJAK2 directs the expression of a hybrid protein consisting of the 98.6 SAb EXT, CD4 TM and Syk CYT domain joined at their C-terminus to the entire mouse JAK2 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.6 kb encoding the entire mJAK2 protein, obtained by digestion of pIKCD4-mJAK2 with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-ζ-mJAK1 with SphI and BamHI, and 3) a fragment of 3.0 kb encoding the remainder of the SAb EXT domain, the CD4 TM domain and the entire Syk protein, obtained by digestion of pIKSAb-Syk-mJAK1 with BamHI and SalI.

c) Construction of SAb-Syk-mJAK3

pIKSAb-Syk-mJAK3 directs the expression of a hybrid protein consisting of the 98.6 SAb EXT, CD4 TM and Syk CYT domain joined at their C-terminus to the entire mouse JAK3 Janus kinase by a Val-Asp dipeptide. This plasmid is

constructed from three DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire mJAK3 protein, obtained by digestion of pIKCD4-mJAK3 with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-ζ-mJAK1 with SphI and BamHI, and 3) a fragment of 3.0 kb encoding the remainder of the SAb EXT domain, the CD4 TM domain and the entire Syk protein, obtained by digestion of pIKSAb-Syk-mJAK1 with BamHI and SalI.

d) Construction of SAb-Syk-hTyk2

pIKSAb-Syk-hTyk2 directs the expression of a hybrid protein consisting of the 98.6 EXT, CD4 TM and Syk CYT domain joined at their C-terminus to the entire human Tyk2 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.5 kb encoding the C-terminus of hTyk2, obtained by digestion of pIKCD4-hTyk2 with EcoRI and BspEI, 2) a 1.6 kb fragment encoding the SAb EXT and part of the CD4 TM domain, obtained by digestion of pIKSAb-mJAK1 with EcoRI and NgoMI, and 3) an 2.3 kb fragment encoding the remainder of the CD4 TM domain, the entire human Syk protein and the N-terminus of hTyk2, obtained by digestion of pIKCD4-Syk-hTyk2 with NgoMI and BspEI.

e) Construction of SAb-Syk-hJAK3

pIKCD4-Syk-hJAK3 directs the expression of a hybrid protein consisting of the 98.6 EXT, CD4 TM and Syk CYT domain joined at their C-terminus to the entire human JAK3 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire hJAK3 protein, obtained by digestion of pIKCD4-hJAK3 with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-ζ-mJAK1 with SphI and BamHI, and 3) a fragment of 3.0 kb encoding the remainder of the SAb EXT domain, the CD4 TM domain and the entire Syk protein, obtained by digestion of pIKSAb-Syk-mJAK1 with BamHI and SalI.

f) Construction of SAb-Syk-hIL2Rβ

pIKSAb-Syk-hIL2Rβ directs the expression of a hybrid protein consisting of the 98.6 EXT, CD4 TM and Syk CYT domain joined at their C-terminus to the human IL2Rβ CYT domain by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 5.0 kb encoding the hIL2Rβ CYT domain, obtained by digestion of pIKCD4-hIL2Rβ with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-ζ-mJAK1 with SphI and BamHI, and 3) a fragment of 3.0 kb encoding the remainder of the SAb EXT domain, the CD4 TM domain and the entire Syk protein, obtained by digestion of pIKSAb-Syk-mJAK1 with BamHI and SalI.

g) Construction of SAb-Syk-hIL2Rγ

pIKSAb-Syk-hIL2Rγ directs the expression of a hybrid protein consisting of the 98.6 EXT, CD4 TM and Syk CYT domain joined at their C-terminus to the human IL2Rγ CYT domain by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 4.4 kb encoding the hIL2Rγ CYT domain, obtained by digestion of pIKCD4-hIL2Rγ with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-ζ-mJAK1 with SphI and BamHI, and 3) a fragment of 3.0 kb encoding the remainder of the SAb EXT domain, the CD4 TM domain and the entire Syk protein, obtained by digestion of pIKSAb-Syk-mJAK1 with BamHI and SalI.

EXAMPLE 7

CPRs containing an intracellular clustering domain: and a Janus kinase or cytokine receptor subunit proliforation signaling domain

Expression vectors for FKBP-Janus kinase and FKBP-cytokine receptor subunit hybrids are created by replacing the CD4 EXT and TM domains in CD4-Janus kinase and CD4-cytokine receptor subunit hybrids with an $(FKBP)_3$ cassette consisting of three repeats of an FKBP module, each of which contains residues 2–108 of FKBP12, the human FK506 binding protein (Standaert et al. (1990) *Nature* 346:671–674). The first FKBP module is preceded by an initiator Met codon, then a two amino linker, Val-Glu. This same Val-Glu dipeptide is found between module 1 & 2 and between modules 2 & 3. The last module is followed by a Val-Asp dipeptide which links it to the first codon of the proliferation signalling domain. Other derivatives lacking the Val-Asp dipeptide or containing other oligo- or polypeptide linkers at the junction of the (FKBP)3 cassette and the Janus kinase or cytokine receptor subunit CYT domain are constructed by oligonucleotide-directed mutagenesis. Still other derivatives of $(FKBP)_3$ lacking the Val-Glu dipeptide linkers or containing other oligo- or polypeptide linkers are constructed by oligonucleotide-directed mutagenesis. The $(FKBP)_3$ cassette is constructed in two steps. First, a plasmid containing the FKBP module, pFKBP, is constructed from two DNA fragment: 1) a vector fragment of 2.9 kb, obtained by digestion of pBluescriptSK (Strategene, La Jolla, Calif.) with XhoI and SalI, and treatment with calf intestine alkaline phosphatase, and 2) a DNA fragment of 0.3 kb encoding the FKBP module, obtained by PCR and digested with XhoI and SalI. The PCR product is prepared using as a template oligo-dT-primed first-strand cDNA made from activated T cell mRNA (as described in Example 1) and oligos nucleotides 27 and 28 (SEQ ID NOS: 27 & 28) as the PCR primers. DNA sequence analysis is employed to confirm the correct structure of the module. Secondly, plasmid $pBSK(FKBP)_3$ containing the $(FKBP)_3$ cassette is constructed from three fragments: 1) a vector fragment of 2.9 kb, obtained by digestion of pBluescriptSK with EcoRI and SalI, 2) a DNA fragment of 1.0 kb encoding $(FKBP)_3$, obtained by extensive self-ligation and subsequent digestion with XhoI and SalI of an 0.3 kb fragment encoding the FKBP module, obtained by digestion of pFKBP with XhoI and SalI, and 3) an EcoRI-XhoI adapter composed of oligos nucleotides 29 and 30 (SEQ ID NOS. 29 & 30).

a) Construction of FKBP-mJAK1

pIKFKBP-mJAK1 directs the expression of a hybrid protein consisting of the $(FKBP)_3$ coding sequence of pBSK $(FKBP)_3$ joined at its C-terminus to the entire mouse JAK1 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 4.3 kb, obtained by digestion of the expression plasmid pIK1.1 with EcoRI and ApaI, 2) a fragment of 1.0 kb encoding the $(FKBP)_3$ cassette, obtained by digestion of $pBSK(FKBP)_3$ with EcoRI and SalI, and 3) a 3.6 kb fragment encoding the entire mJAK1 protein, obtained by digestion of pIKCD4-mJAK1 with SalI and ApaI.

b) Construction of FKBP-mJAK2

pIKFKBP-mJAK2 directs the expression of a hybrid protein consisting of the $(FKBP)_3$ coding sequence of pBSK $(FKBP)_3$ joined at its C-terminus to the entire mouse JAK2 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 7.6 kb encoding the entire mJAK2 protein, obtained by digestion of pIKCD4-mJAK2 with SphI and SalI, and 2) a fragment of 1.1 kb encoding the $(FKBP)_3$ cassette, obtained by digestion of pIKFKBP-mJAK1 with SphI and SalI.

c) Construction of FKBP-mJAK3

pIKFKBP-mJAK3 directs the expression of a hybrid protein consisting of the $(FKBP)_3$ coding sequence of pBSK

$(FKBP)_3$ joined at its C-terminus to the entire mouse JAK3 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire mJAK3 protein, obtained by digestion of pIKCD4-mJAK3 with SphI and SalI, and 2) a fragment of 1.1 kb encoding the $(FKBP)_3$ cassette, obtained by digestion of pIKFKBP-mJAK1 with SphI and SalI.

d) Construction of FKBP-hTyk2

pIKFKBP-hTyk2 directs the expression of a hybrid protein consisting of the $(FKBP)_3$ coding sequence of pBSK $(FKBP)_3$ joined at its C-terminus to the entire human Tyk2 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.5 kb encoding the C-terminus of hTyk2, obtained by digestion of pIKCD4-hTyk2 with EcoRI and BspEI, 2) a fragment of 1.0 kb encoding the $(FKBP)_3$ cassette, obtained by digestion of pIKFKBP-mJAK1 with EcoRI and SalI, and 3) a fragment of 0.3 kb encoding the N-terminus of the hTyk2 protein, obtained by digestion of pIKCD4-hTyk2 with SalI and BspEI.

e) Construction of FKBP-hJAK3

pIKFKBP-hJAK3 directs the expression of a hybrid protein consisting of the $(FKBP)_3$ coding sequence of pBSK $(FKBP)_3$ joined at its C-terminus to the entire human JAK3 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire hJAK3 protein, obtained by digestion of pIKCD4-hJAK3 with SphI and SalI, and 2) a fragment of 1.1 kb encoding the $(FKBP)_3$ cassette, obtained by digestion of pIKFKBP-mJAK1 with SphI and SalI.

f) Construction of FKBP-IL2Rβ

pIKFKBP-hIL2Rβ directs the expression of a hybrid protein consisting of the $(FKBP)_3$ coding sequence of pBSK $(FKBP)_3$ joined at its C-terminus to the human IL2Rβ CYT domain by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 5.0 kb encoding the hIL2Rβ CYT domain, obtained by digestion of pIKCD4-hIL2Rβ with SphI and SalI, and 2) a fragment of 1.1 kb encoding the $(FKBP)_3$ cassette, obtained by digestion of pIKFKBP-mJAK1 with SphI and SalI.

g) Construction of FKBP-IL2Rγ

pIKFKBP-hIL2Rγ directs the expression of a hybrid protein consisting of the $(FKBP)_3$ coding sequence of pBSK $(FKBP)_3$ joined at its C-terminus to the human IL2Rγ CYT domain by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 4.4 kb encoding the hIL2Rγ CYT domain, obtained by digestion of pIKCD4-hIL2Rγ with SphI and SalI, and 2) a fragment of 1.1 kb encoding the $(FKBP)_3$ cassette, obtained by digestion of pIKFKBP-mJAK1 with SphI and SalI.

EXAMPLE 8

CPRs containing a ligand-receptor (CD4) extracellular clustering domain; an intracellular clustering domain; and a Janus kinase or cytokine receptor subunit proliferation signaling domain

This class of chimeric receptors are created by the insertion of an $(FKBP)_3$ cassette into a CD4-Janus kinase or CD4-cytokine receptor subunit CPR between the TM domain and proliferation signaling domain. These chimeric receptors are constructed from pIKCD4-$(FKBP)_3$, an intermediate plasmid based on pIK1.1F3Sal. The proliferation signaling domain of a Janus kinase or cytokine receptor subunit is then joined at the C-terminus of CD4-$(FKBP)_3$ using the unique SalI site which adds a Val-Asp dipeptide at the junction. Derivatives lacking the Val-Asp dipeptide or containing other oligo- or polypeptide linkers are constructed by oligonucleotide-directed mutagenesis. A similar strategy is used to create CPRs containing an $(FKBP)_3$ cassette at the C-terminus of the chimeric protein (e.g., CD4-Janus kinase-FKBP and CD4-cytokine receptor subunit-FKBP) by inserting the $(FKBP)_3$ cassette after the proliferation signalling CYT domain. pIKCD4-$(FKBP)_3$ is constructed from two DNA fragments: 1) a vector fragment of 5.8 kb encoding the CD4 EXT and TM domains, obtained by digestion of pIK1.1F3Sal with SalI followed by treatment with calf intestine alkaline phosphatase, and 2) a 1.0 kb fragment encoding the $(FKBP)_3$ cassette, obtained by digestion of pBSK$(FKBP)_3$ with XhoI and SalI. Clones with the $(FKBP)_3$ cassette in the correct in-frame orientation are confirmed by restriction mapping.

a) Construction of CD4-FKBP-mJAK1

pIKCD4-FKBP-mJAK1 directs the expression of a hybrid protein consisting of the CD4-$(FKBP)_3$ coding sequence joined at its C-terminus to the entire mouse JAK1 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire mJAK1, obtained by digestion of pIKCD4-mJAK1 with SphI and SalI, and 2) a fragment of 2.3 kb encoding CD4-$(FKBP)_3$, obtained by digestion of pIKCD4-$(FKBP)_3$ with SphI and SalI.

b) Construction of CD4-FKBP-mJAK2

pIKCD4-FKBP-mJAK2 directs the expression of a hybrid protein consisting of the CD4-$(FKBP)_3$ coding sequence joined at its C-terminus to the entire mouse JAK2 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 7.6 kb encoding the entire mJAK2, obtained by digestion of pIKCD4-mJAK2 with SphI and SalI, and 2) a fragment of 2.3 kb encoding CD4-$(FKBP)_3$, obtained by digestion of pIKCD4-$(FKBP)_3$ with SphI and SalI.

c) Construction of CD4-FKBP-mJAX3

pIKCD4-FKBP-mJAK3 directs the expression of a hybrid protein consisting of the CD4-$(FKBP)_3$ coding sequence joined at its C-terminus to the entire mouse JAK3 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire mJAK3, obtained by digestion of pIKCD4-mJAK3 with SphI and SalI, and 2) a fragment of 2.3 kb encoding CD4-$(FKBP)_3$, obtained by digestion of pIKCD4-$(FKBP)_3$ with SphI and SalI.

d) Construction of CD4-FKBP-hTyk2

pIKCD4-FKBP-hTyk2 directs the expression of a hybrid protein consisting of the CD4-$(FKBP)_3$ coding sequence joined at its C-terminus to the entire human Tyk2 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.5 kb encoding the C-terminus of hTyk2, obtained by digestion of pIKCD4-hTyk2 with EcoRI and BspEI, 2) a fragment of 2.3 kb encoding the CD4-$(FKBP)_3$ cassette, obtained by digestion of pIKCD4-$(FKBP)_3$ with EcoRI and SalI, and 3) a fragment of 0.3 kb encoding the N-terminus of the hTyk2 protein, obtained by digestion of pIKCD4-hTyk2 with SalI and BspEI.

e) Construction of CD4-FKBP-hJAK3

pIKCD4-FKBP-hJAK3 directs the expression of a hybrid protein consisting of the CD4-$(FKBP)_3$ coding sequence joined at its C-terminus to the entire human JAK3 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire hJAK3, obtained by digestion of pIKCD4-hJAK3 with SphI and SalI, and 2) a fragment of 2.3 kb encoding CD4-$(FKBP)_3$, obtained by digestion of pIKCD4-$(FKBP)_3$ with SphI and SalI.

f) Construction of CD4-FKBP-IL2Rβ

pIKCD4-FKBP-hIL2Rβ directs the expression of a hybrid protein consisting of the CD4-$(FKBP)_3$ coding sequence joined at its C-terminus to the hIL2Rβ CYT domain by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 5.0 kb encoding the hIL2Rβ CYT domain, obtained by digestion of pIKCD4-hIL2Rβ with SphI and SalI, and 2) a fragment of 2.3 kb encoding CD4-$(FKBP)_3$, obtained by digestion of pIKCD4-$(FKBP)_3$ with SphI and SalI.

g) Construction of CD4-FKBP-IL2Rγ

pIKCD4-FKBP-hIL2Rγ directs the expression of a hybrid protein consisting of the CD4-$(FKBP)_3$ coding sequence joined at its C-terminus to the hIL2Rγ CYT domain by a Val-Asp dipeptide. This plasmid is constructed from two DNA fragments: 1) a vector fragment of 4.4 kb encoding the entire mJAK1, obtained by digestion of pIKCD4-hIL2Rγ with SphI and SalI, and 2) a fragment of 2.3 kb encoding CD4-$(FKBP)_3$, obtained by digestion of pIKCD4-$(FKBP)_3$ with SphI and SalI.

EXAMPLE 9

CPRs containing antibody extracellular clustering domain, an intracellular clustering domain: and a Janus kinase or cytokine receptor subunit proliferation domain

This class of chimeric receptors are created by the insertion of an $(FKBP)_3$ cassette into a SAb-Janus kinase or SAb-cytokine receptor subunit CPR between the TM domain and proliferation signalling domain. The proliferation signalling domain of a Janus kinase or cytokine receptor subunit is joined at the C-terminus of SAb-$(FKBP)_3$ using the SalI site which adds a Val-Asp dipeptide at the junction. Derivatives lacking the Val-Asp dipeptide or containing other oligo- or polypeptide linkers are constructed by oligonucleotide-directed mutagenesis. A similar strategy is used to create CPRs containing an $(FKBP)_3$ cassette at the C-terminus of the chimeric protein (e.g., SAb-Janus kinase-FKBP and SAb-cytokine receptor subunit-FKBP) by inserting the $(FKBP)_3$ cassette after the proliferation signalling CYT domain.

a) Construction of SAb-FKBP-mJAK1

pIKSAb-FKBP-mJAK1 directs the expression of a hybrid protein consisting of the SAb EXT domain, CD4 TM domain and $(FKBP)_3$ cassette joined to the entire mouse JAK1 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire mJAK1 protein, obtained by digestion of pIKCD4-mJAK1 with SphI and SalI, 2) a fragment of 17 kb encoding the SAb EXT domain and a portion of the CD4 TM domain, obtained by digestion of pIKSAb-mJAK1 with SphI and NgoMI, and 3) a 1.0 kb fragment encoding the remainder of the CD4 TM domain and the $(FKBP)_3$ cassette, obtained by digestion of pIKCD4-$(FKBP)_3$ with NgoMI and SalI.

b) Construction of SAb-FKBP-mJAK2

pIKSAb-FKBP-mJAK2 directs the expression of a hybrid protein consisting of the SAb EXT domain, CD4 TM domain and $(FKBP)_3$ cassette joined to the entire mouse JAK2 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.6 kb encoding the entire mJAK2 protein, obtained by digestion of pIKCD4-mJAK2 with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-mJAK1 with SphI and BamHI, and 3) a fragment of 2.0 kb encoding the remainder of the SAb EXT domain, the CD4 TM domain and the $(FKBP)_3$ cassette, obtained by digestion of pIKSAb-FKBP-mJAK1 with BamHI and SalI.

c) Construction of SAb-FKBP-mJAK3

pIKSAb-FKBP-mJAK3 directs the expression of a hybrid protein consisting of the SAb EXT domain, CD4 TM domain and $(FKBP)_3$ cassette joined to the entire mouse JAK3 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire mJAK3 protein, obtained by digestion of pIKCD4-mJAK3 with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-mJAK1 with SphI and BamHI, and 3) a fragment of 2.0 kb encoding the remainder of the SAb EXT domain, the CD4 TM domain and the $(FKBP)_3$ cassette, obtained by digestion of pIKSAb-FKBP-mJAK1 with BamHI and SalI.

d) Construction of SAb-FKBP-hTyk2

pIKSAb-FKBP-hTyk2 directs the expression of a hybrid protein consisting of the SAb EXT domain, CD4 TM domain and $(FKBP)_3$ cassette joined to the entire human Tyk2 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.5 kb encoding the C-terminus of the Tyk2 protein, obtained by digestion of pIKCD4-hTyk2 with EcoRI and BspEI, 2) a fragment of 1.6 kb encoding the SAb EXT domain and a portion of the CD4 TM domain, obtained by digestion of pIKSAb-mJAK1 with EcoRI and NgoMI, and 3) a fragment of 1.5 kb encoding the remainder of the CD4 TM domain, the $(FKBP)_3$ cassette and the N-terminus of hTyk2, obtained by digestion of pIKCD4-FKBP-hTyk2 with NgoMI and BspEI.

e) Construction of SAb-FKBP-hJAK3

pIKSAb-FKBP-hJAK3 directs the expression of a hybrid protein consisting of the SAb EXT domain, CD4 TM domain and $(FKBP)_3$ cassette joined to the entire human JAK3 Janus kinase by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 7.7 kb encoding the entire mJAK2 protein, obtained by digestion of pIKCD4-hJAK3 with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-mJAK1 with SphI and BamHI, and 3) a fragment of 2.0 kb encoding the remainder of the SAb EXT domain, the CD4 TM domain and the $(FKBP)_3$ cassette, obtained by digestion of pIKSAb-FKBP-mJAK1 with BamHI and SalI.

f) Construction of SAb-FKBP-IL2Rβ

pIKSAb-FKBP-hIL2Rβ directs the expression of a hybrid protein consisting of the SAb EXT domain, CD4 TM domain and $(FKBP)_3$ cassette joined to the hIL2Rβ CYT domain by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 5.0 kb encoding the hIL2Rβ CYT domain, obtained by digestion of pIKCD4-hIL2Rβ with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-mJAK1 with SphI and BamHI, and 3) a fragment of 2.0 kb encoding the remainder of the SAb EXT domain, the CD4 TM domain and the $(FKBP)_3$ cassette, obtained by digestion of pIKSAb-FKBP-mJAK1 with BamHI and SalI.

g) Construction of SAb-FKBP-IL2Rγ

pIKSAb-FKBP-hIL2Rγ directs the expression of a hybrid protein consisting of the SAb EXT domain, CD4 TM domain and $(FKBP)_3$ cassette joined to the hIL2Rγ CYT domain by a Val-Asp dipeptide. This plasmid is constructed from three DNA fragments: 1) a vector fragment of 4.4 kb encoding the hIL2Rγ CYT domain, obtained by digestion of pIKCD4-hIL2Rγ with SphI and SalI, 2) a fragment of 0.7 kb encoding the N-terminal portion of the SAb EXT domain, obtained by digestion of pIKSAb-mJAK1 with SphI and

BamHI, and 3) a fragment of 2.0 kb encoding the remainder of the SAb EXT domain, the CD4 TM domain and the $(FKBP)_3$ cassette, obtained by digestion of pIKSAb-FKBP-mJAK1 with BamHI and SalI.

EXAMPLE 10

Expression of CPRs

To determine whether CPR polypeptides can be expressed and properly folded, each construct was initially transfected into a model mammalian cell, the human 293 embryonic kidney cell line (ATCC CRL1573). Following transfection, the expression of each construct was evaluated by radioimmunoprecipitation, and its transport to the cell surface (for CPRs comprising a ligand-receptor or antibody EXT domain) was evaluated by fluorescent-activated cell sorting (FACS) analysis.

a) Transfection of human 293 cells with CPR expression vectors CPRs were constructed in pIK mammalian expression plasmids as described and transfected into human 293 cells. 293 cells were grown in complete DMEM (JRH Biosciences, Lenexa, Kans.), 1 g/l glucose, 10% donor calf serum (JRH Biosciences) and passaged at 1:10 split ratio every 3 days. Twenty-four hours prior to transfection, 293 cells were plated at 5×10^5 cells per 10 cm plate. Ten micrograms of plasmid DNA was transfected onto a 10 cm dish of 293 cells by the calcium phosphate coprecipitation method (Wigler et al. (1979) *Cell* 16:777). Twenty-four hours after transfection, the cells were fed with fresh complete DMEM media. The expression of CPRs was evaluated by FACS analysis and radioimmunoprecipitation at 48 hours post-transfection.

b) FACS analysis of CPR expression in 293 cells.

Transfected 293 cells were rinsed once with PBS and incubated in 150 mM NaCl, 40 mM Tris-HCl pH7.5, 1 mM EDTA solution for 5 minutes at room temperature. Cells were collected from plates, centrifuged and resuspended in PBS/1% FCS. Approximately 1×10^6 cells/sample were stained directly with saturating concentrations of a fluorescein (FITC)-conjugated anti-CD4 monoclonal antibody (MAb) (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). Mouse FITC-IgG1 and PE-IgG2a were used as negative control MAbs. 293 cells transfected with 10 μg of PIKF3, which expresses CD4-ζ, were used as a positive control. All FACS analyses were performed in a FACScan (Becton Dickinson) as previously described (Weiss and Stobo, (1984) *J. Exp. Med.*, 160:1284–1299). FACS analysis of cells transfected with CPRs containing a CD4 EXT clustering domain demonstrated that up to 50% of cells were stained positive with the anti-CD4 MAb (FIG. 3(A)–(L)). 293 cells transfected with CPR constructs containing a SAb EXT clustering domain are evaluated for expression of the CPR by staining with a fluorescein-conjugated mouse anti-human Ig MAb, using isotype-matched mouse FITC-IgG as a negative control. 293 cells transfected with CPR constructs containing an intracellular clustering domain (e.g., FKBP, glucocorticoid receptor) are evaluated for expression of the CPR by first partially permeabilizing the cells with 70% methanol for 30 seconds on ice, followed by staining the cells with FITC-conjugated anti-PSD antibody (see Example 10C). An isotype matched mouse FITC-IgG is used as a negative control.

c) Radioimmunoprecipitation of CPRs expressed in 293 cells

Transfected 293 cells were rinsed once with RPMI medium lacking methionine. Cells were cultured for additional 8 hours in 2 μl of methionine-deficient RPMI supplemented with 200 μCi [^{35}S]-methionine (1160 C/mmol, ICN Biomedicals, Inc., Irvine, Calif.). The labelled cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X 100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS)). For immunoprecipitation, cell lysates were precleared with 10 μl Pansorbin (Calbiochem, La Jolla, Calif.) and incubated with either OKT4A (anti-CD4) (Ortho Diagnostic Systems, Raritan, N.J.), polyclonal anti-mouse/human JAK1 (UBI, Lake Placid, N.Y.), polyclonal anti-mouse JAK2 (UBI), or polyclonal anti-mouse JAK3 (UBI), at 4° C. for 1 hour. Ten microliters of Pansorbin was then added to the lysates to precipitate the antibody-bound antigen. Immunoprecipitates were washed three times in RIPA buffer, boiled in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and analyzed by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were fixed in 20% methanol/10% acetic acid and soaked in Enlightening solution (NEN Research Products, Boston, Mass.) for 15 min, dried and subjected to autoradiography. SDS-PAGE analysis revealed the expression of CPRs in 293 cells of the expected molecular mass (FIG. 4)

EXAMPLE 11

Biochemical and biological properties of CPRs expressed in human $CD8^+$ T cells

a) Construction of CPR-expressing retroviral vectors

Sequences encoding the CPRs CD4-mJAK1, CD4-ζ-mJAK1, CD4-mJAK3, CD4-ζ-mJAK3, CD4-hTyk2, and CD4-ζ-hTyk2 were inserted between the EcoRI and ApaI sites in pIK1.1, and were subsequently excised and inserted between analogous EcoRI and ApaI sites of pRT43.2F3, described in U.S. patent application Ser. No. 08/258,152 incorporated herein in its entirety by reference, generally as two subfragments to avoid internal EcoRI or ApaI sites within the CPR constructs. One skilled in the art can readily devise schemes for producing retroviral vectors containing other CPRs.

b) Infection of human $CD8^+$ T cells with CPR-expressing retroviral vectors

Human $CD8^+$ T lymphocytes were isolated from peripheral blood lymphocytes (PBL) obtained from healthy donors by purification with the CEPRATE LC system (CellPro, Inc., Bothell, Wash.), followed by negative selection against $CD4^+$ cells using a T-25 MicroCELLector (AIS, Inc., Santa Clara, Calif.). The final purified cell population contained greater than 98% $CD8^+$ cells according to FACS analysis. Immediately after purification, cells were stimulated for 24 hours with an equal number of γ-irradiated autologous PBMCs in AIM-V media (GibcoBRL, Grand Island, N.Y.) containing 10 ng/μl of OKT3 MAb and 100 units of human IL-2 (Chiron Corp., Emeryville, Calif.). Cells were then washed free of OKT3 and cultured in AR media (50% AIM-V, 50% RPMI, 4 mM Glutamine, 20 mM Hepes, 1 mM Na-Pyruvate, non-essential amino acids, and 100 units human IL-2) supplemented with 5% heat inactivated human AB plasma (Sigma, St. Louis, Mo.). Retrovirus was prepared in the TIN-4 cell line derived from thymidine kinase-expressing human 293 cells. For the transduction of human $CD8^+$ cells, TIN-4 cells were seeded at 5×10^5 cell/plate in 6-well plates (Corning Glass, Corning, N.Y.) in complete DMEM medium 48 hours prior to transfection. Ten micrograms of CPR construct in the retroviral vector pRT43.2 were transfected per plate in the absence or presence of packaging plasmids by the calcium phosphate coprecipitation method. Following transfection, 1.5 ml of fresh AR medium containing 100 units/ml of human IL-2 was added to each well of the plate. Three hours later, 5×10^5 of $CD8^+$ T cells in AR media containing 100 units/ml of human IL-2 and 2 μg/ml of polybrene were added to each well of the

plate. CD8+ T cells were removed from the 6-well plates 24 hours later and then transduced a second time by the same procedure. Newly transduced CD8+ T cells were maintained in AR media.

c) FACS analysis of CPR expression in human CD8+ T cells

At various times following transduction, CD8+ T cells were harvested and washed with PBS/1% FCS. Approximately 1×10^6 CD8+ T cells were stained with specific antibodies for FACS analysis as described in Example 10B. As shown in Table 1, chimeric proliferation receptors can be expressed on the surface of CD8+ T cells.

TABLE I

Transduction	% Positive in CD8+ T Cells
Mock	1.7
CD4-ζ	18.2
CD4-mJAK1	4.0
CD4-mJAK3	3.8
CD4-hTyk2	7.5
CD4-ζ-hTyk2	4.6

d) Immunoprecipitation analysis of CPR expression in human CD8+ T cells

At various times following transduction, human CD8+ T cells are harvested and placed in methionine-depleted AR media supplemented with 200 μCi [^{35}S]-methionine (1160 Ci/mmol, ICN Biomedicals, Inc.). Cells are lysed in RIPA buffer, precleared with 10 μl Pansorbin (except cells expresssed SAb-containing CPRs) (Calbiochem, La Jolla, Calif.), and then incubated with either OKT4A (Ortho Diagnostic Systems), polyclonal anti-mouse/human JAK1 (UBI, Lake Placid, N.Y.), polyclonal anti-mouse JAK2 (UBI), or polyclonal anti-mouse JAK3 (UBI) at 4° C. for 1 hour. Ten microliters of Pansorbin are then added to the lysates to precipitate the antibody-bound antigen. The immunoprecipitates are washed three times in RIPA buffer, boiled in SDS sample buffer and analyzed by 7.5% SDS-polyacrylamide gel electrophoresis. Gels are fixed in 20% methanol/ 10% acetic acid and then soaked in Enlightening solution (NEN Research Products, Boston, Mass.) for 15 minutes, dried and subjected to autoradiography. SDS-PAGE analysis reveals the molecular mass of CPRs expressed in human CD8+ T cells.

e) Analysis of CPR-expressing human CD8+ T cells for phosphotyrosine content

To assess the phosphotyrosine content of human CD8+ T cells expressing CPRs, 5×10^6 cells are lysed in protein phosphotyrosine lysis buffer (1% Nonidet P-40, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 100 uM orthovanadate) at 4° C. for 15 min, and immunoprecipitated with either OKT4A, anti-human/mouse JAK1, anti-mouse JAK2, anti-mouse JAK3, anti-human JAK3 or anti-human-Tyk2. The immunoprecipitates are separated by 7.5% SDS-PAGE and the proteins are transferred electrophoretically to a nitrocellulose membrane in transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol, 0.2% SDS) at 50 volts for 4 hours. Membranes are blocked in TBST (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween-20) containing 1% BSA and then incubated with primary anti-phosphotyrosine antibody 4G10 (UBI). The membrane is developed using the enhanced chemiluminescence (ECL) detection system (Amersham, Arlington Height, Ill.).

f) Analysis of CPR-expressing human CD8+ T cell lysates for in vitro kinase activity

As JAK kinases have the ability to be autophosphorylated, human CD8+ T cells expressing CPRs are evaluated for their CPR-associated tyrosine kinase activity. Immunoprecipitates prepared from CPR-transduced human CD8+ T cells using either OKT4A, anti-human Fc Mab, anti-human/mouse JAK1, anti-mouse JAK2, anti-mouse JAK3, anti-human JAK3 or anti-human-Tyk2, as described above, are washed three times with protein tyrosine lysis buffer and once with kinase buffer (10 mM $MnCl_2$, 50 mM Tris-HCl, pH 7.5). Kinase reactions are performed in 25 μl of kinase buffer containing 10 μCi γ-[32-P]ATP (95,000 Ci/mmole, Amersham). Following a 5 minute incubation at 25° C., the reactions are terminated by addition of equal volume of 2×SDS sample buffer, boiled for 5 minutes and subjected to SDS-PAGE. The gel is fixed, treated with 1M KOH at 55° C. for 1 hour to remove serine/threonine phosphorylated residues, refixed, dried and subjected to autoradiography.

g) Proliferative response of CPR-expressing human CD8+ T cells

To evaluate the ability of CPR-expressing CD8+ T cells to proliferate in an antigen-driven or inducer molecule-driven fashion, cells are first rested by serum starvation for 16 hours. Cells are then placed in culture dishes coated with saturating concentrations of either OKT4A, anti-human Fc Mab, gp120, gp160-expressing cells, gp41/gp120-expressing cells, HIV-1 infected cells or FK1012. After 5 to 48 hours, the total cell numbers is determined by counting, following staining with trypan blue/PBS. The cell number is compared with the original cell number, and the cell numbers obtained after starvation with or without stimulation with media containing human serum. In addition, analysis of cellular proliferation is carried out by measuring radioactive thymidine incorporation. Cells are starved for 16 hours and aliquoted in quadruplicate into microliter plates at 5×10^4 cells/well. The plates are either coated with OKT4A or anti-gp120, gp160-expressing cells, gp41/gp120-expressing cells, HIV-1 infected cells or FK1012. Cells are cultured under these conditions for up to three days, and thymidine incorporation is measured in a liquid scintillation counter after pulsing the cells for the last 8 hours with 1 μCi/well of [^{3}H]thymidine (NEN Corp, Boston, Mass.).

h) C-myc induction in CPR-expressing human CD8+ T cells

To evaluate the induction of the c-myc proto-oncogene in CPR-expressing CD8+ T cells stimulated with a specific antigen or inducer molecule, mRNA is prepared using a Fast Track mRNA isolation kit (Invitrogen, San Diego, Calif.). Two micrograms of mRNA is denatured with formaldehyde/formamide and run on a 1% agarose-formaldehyde gel as described (Sambrook et al, *Molecular Cloning*, Cold Spring Harbor Laboratory Press, 1989). The mRNA is transferred overnight by capillary action to a nitrocellulose membrane (Schleicher and Schuell, Keene, N.H.) in 10× SSC buffer. The membrane is hybridized overnight with a c-myc probe at 65° C. in 6× SSC, 0.5% sodium dodecyl sulfate and 100 mg/ml of denatured herring sperm DNA, washed in 0.2× SSC and subjected to autoradiography. The c-myc probe is prepared with a 1 kb ClaI-EcoRI fragment obtained from pMyc6514 (Battey et al, *Cell* 34, 779–787, 1983) which contains the third exon of human c-myc. Radiolabelling of the probe is carried by random priming with *E. coli* DNA polymerase, dNTPs and α [32-P]dCTP (3000 Ci/mmole, Amersham, Arlington Heights, Ill.) as described (Sambrook et al). As a control for the amount of RNA loaded on the gel, the nitrocellulose membrane is rehybridized with a 1.3 kb mouse β-actin probe (Stratagene, La Jolla, Calif.). A PhosphoImager (Molecular Devices, Menlo Park, Calif.) is used to quantitate the amount of probe bound to the membrane.

i) Calcium mobilization response in CPR-expressing human CD8+ T cells

The mobilization of intracellular [Ca^{2+}] by CPR-expressing human CD8+ T cells is measured using Indo-1 acetomethoxyester (Molecular Probes, Eugene, Oreg.) on a FACStar Plus (Beckton Dickinson). Cells are collected by centrifugation, resuspended at 3×10^6/ml in complete medium containing 1 mM Indo-1 (Grynkiewicz et al., (1985) *J. Biol. Chem.* 260:3440–3450) and incubated at 37° C. for 45 min. The Indo-1-loaded cells are pelleted and resuspended at 1×10^6/ml in serum-free medium. Cells are then stimulated by treatment with either saturating levels of OKT4A or anti-human Fc Mab and cross-linking goat anti-mouse IgG, gp120, gp160-expressing cells, HIV-1 infected cells or FK1012, and fluorescence is measured. Maximal fluorescence is determined after lysis of cells with Triton X-100; minimal fluorescence is obtained after chelation of Ca^{2+} with EGTA. Intracellular [Ca^{2+}] is determined using the following equation: $[Ca^{2+}]=K_d(F_{observed}-F_{min})/(F_{max}-F_{observed})$, with K_d=250 nM as described (Grynkiewicz, 1985).

j) Cytolytic activity of CPR-expressing human CD8+ T cells

To determine the cytolytic activity of CPR-expressing human CD8+ T cells, in vitro cytolytic assays are carried out with target cells expressing HIV-1 antigens. Gp160-expressing 293 cells or HIV-1 infected human T cells are labeled at 37° C. overnight with 10μCi [^{3}H]TdR (Roberts et al., *Blood* 84:2878–2889 (1994)), washed and aliquoted to 96-well V-bottom plates at 1×10^4/well. Serial dilutions of CPR-expressing human CD8+ T cells are made to achieve an effector to target (E:T) ratio ranging from 100:1 to 0.1:1. Sample are set up in triplicate and incubations are carried out for 6 hours at 37° C. Following incubation, aliquots of the culture supernatant are removed and counted in a liquid scintillation counter. Spontaneous release (SR) is obtained in a negative control sample lacking CPR-expressing human CD8+ T cells; maximum release (MR) is obtained from a positive control sample by lysing target cells with 1N HCl. The percent specific lysis is calculated from the following equation:

$$\%\ \text{specific lysis}=(SR_{cpm}-Sample_{cpm})/(Sample_{cpm}-MR_{cpm})\times100\%.$$

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 31

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCTGCTGAAC TTCACTCTGT CGACACAGAA GAAGATGCC 39

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCGACATGCA GTATCTAAAT ATAAAAGAGG ACTGCAATGC 40

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CATGGCATTG CAGTCCTCTT TTATATTTAG ATACTGCATG 40

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TATGTGTCAG TGGGGCGGGC C 21

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGCCCCACTG ACACA 15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTAAGGCAGG CCATTCCCAT GTCGACACAG AAGAAGATGC C 41

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCTGTGTCGA CATGGG 16

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

-continued

TCGACATGGC ACCTCCAAGT GAGGAGACAC CTCTGATCCC TCAGC 45

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCTGAGGGAT CAGAGGTGTC TCCTCACTTG GAGGTGCCAT G 41

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GATCCCTAGT TTATTCATGG GCC 23

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CATGAATAAA CTAGG 15

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CATCCCCCAG TGGCGCAGAG GCATGTCGAC AGAGTGAAGT TC 42

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTCGACATGC CTCTGC 16

(2) INFORMATION FOR SEQ ID NO:14:

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGGCCGCCGG AATTCCATGT CGACACAGAA GAAGATGCC 39

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCTGTGTCGA CATGGA 16

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CCTCAACAGG GTCCTTC 17

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCTGATCGTC GACAACTGCA GGAACACCGG 30

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CATCTGTGAT ATCTCTACAC CAAGTGAGTT G 31

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GAAGAGCAAG CGCCATGTTG AAGCCATCAT TACCATTCAC 40

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AGCCTGAAAC CTGAACCCCA ATCCTCTGAC AGAAGAACCC 40

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CTGGCTGGTC GACGAACGGA CGATGCCCCG CATTCCCACC CTGAAGAAC 49

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GATTGGGGGA TATCTCAGGT TTCAGGCTTT AG 32

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GAAATCCCCT GGCTGTTAGT CGACGCGAGG GGGCAGGGCC TG 42

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:24:

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TGTTAGTCGA CGCGAG 16

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GGTCCACTCG AGATGGCCAG CAGCGGCATG 30

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCAGGTCCGA TATCTTAGTC GACGTTCACC ACGTCATAGT A 41

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GACTGACTCT CGAGGGCGTG CAGGTGGAAA CC 32

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GACTGACTGT CGACTTCCAG TTTTAGAAGC TC 32

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:29:

AATTCAAGGC CACAATGC 18

(2) INFORMATION FOR SEQ ID NO:30:

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TCGAGCATTG TGGCCTTG 18

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys Gly
1 5 10

What is claimed is:

1. A method of inducing a cell to proliferate comprising:

(a) introducing a proliferation receptor construct into said cell under conditions suitable for expression to produce a cell expressing a proliferation receptor protein; and

(b) contacting said receptor in said cell with an inducer, said construct comprising in reading frame:

a DNA sequence encoding an intracellular inducer-responsive clustering domain comprising an immunophilin or a cyclophilin; and

a DNA sequence encoding a proliferation signaling domain comprising a Janus tyrosine kinase.

2. The method of claim 1 wherein said cell is selected from the group consisting of a nerve cell, a keratinocyte cell, islet of Langerhans cell, a muscle cell, or a hematopoietic cell.

3. A method of inducing a cell to proliferate comprising:

(a) introducing a proliferation receptor construct into said cell under conditions suitable for expression to produce a cell expressing a proliferation receptor protein; and

(b) contacting said receptor in said cell with an inducer, said construct comprising in reading frame:

a DNA sequence encoding a signal sequence;

a DNA sequence encoding an extracellular inducer-responsive clustering domain that binds specifically to at least one inducer molecule which results in the dimerization or oligomerization of said extracellular domain;

a DNA sequence encoding a transmembrane domain; and

a DNA sequence encoding a proliferation signaling domain comprising a Janus tyrosine kinase.

4. The method of claim 3, wherein said extracellular domain is an antibody, a single chain antibody or an antigen binding fragment of said antibody.

5. The method of claim 4, wherein said extracellular domain is CD8 or CD4.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,837,544

DATED : November 17,1998

INVENTOR(S) : Daniel J. Capon, Huan Tian, Douglas H. Smith, Genine A. Winslow and Miriram Siekevitz

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Claim 5, line 1, delete "claim 4" and insert --claim 3--.

Signed and Sealed this

Twentieth Day of July, 1999

Attest:

Attesting Officer

Q. TODD DICKINSON

Acting Commissioner of Patents and Trademarks

US006103521A

United States Patent [19]

Capon et al.

[11] Patent Number: 6,103,521

[45] Date of Patent: Aug. 15, 2000

[54] MULTISPECIFIC CHIMERIC RECEPTORS

[75] Inventors: **Daniel J. Capon**, Hillsborough; **Douglas H. Smith**, Foster City; **Huan Tian**, Cupertino; **Genine A. Winslow**, Hayward, all of Calif.; **Miriam Siekevitz**, New York, N.Y.

[73] Assignee: **Cell Genesys, Inc.**, Foster City, Calif.

[21] Appl. No.: **08/454,098**

[22] Filed: **May 30, 1995**

Related U.S. Application Data

[63] Continuation of application No. 08/384,033, Feb. 6, 1995, abandoned.

[51] **Int. Cl.**[7] **C12N 15/62**; C07K 14/705

[52] **U.S. Cl.** **435/325**; 435/69.7; 435/320.1; 530/350; 530/387.3; 536/23.4

[58] **Field of Search** 536/23.4; 435/69.1, 435/240.2, 252.3, 320.1, 325, 69.7; 530/350, 351, 387.1, 387.3

[56] **References Cited**

U.S. PATENT DOCUMENTS

5,260,203	11/1993	Ladner et al.	435/172.3
5,336,603	8/1994	Capon et al.	435/69.7
5,359,046	10/1994	Capon et al.	536/23.4
5,741,899	4/1998	Capon et al.	536/23.4

FOREIGN PATENT DOCUMENTS

0340793	5/1988	European Pat. Off. .
0610046	8/1994	European Pat. Off. .
WO 88/09344	12/1988	WIPO .
WO 91/04329	4/1991	WIPO .
9210591	6/1992	WIPO .
9215322	9/1992	WIPO .
WO 93/02198	2/1993	WIPO .
WO 93/11161	6/1993	WIPO .
WO9319163	9/1993	WIPO .
WO 94/04691	3/1994	WIPO .
WO 94/12520	6/1994	WIPO .
WO 94/13806	6/1994	WIPO .
WO 94/22914	10/1994	WIPO .
9502686	1/1995	WIPO .

OTHER PUBLICATIONS

Bird et al., J.Biol.Chem. 265:19151–19157, (1990).
Caruso, Mol.Med.Today 2:181–223, (1996).
Coghlan, New Scientist, Nov., (1995).
Culver et al., TIG 10:174–178, (1994).
Stein et al., Cell Biol. 14:3392–3402, (1994).
Gorny et al., J.Virol. 66:7538–7542, (1992).
Gorochov et al., Int.J.Cancer 7:53–57, (1992).
Gross et al., Proc.Natl.Acad.Sci. 86:10024–10028, (1989).
Gunzburg et al., Mol.Med.Today pp. 410–417, (1995).
Heinzel et al., J.Virol. 62:3738–3746, (1988).
Olshevsky et al., J.Virol. 64:5701–5707, (1990).
Hoffman–LaRoche, Curr.Opin.Ther.Pat. 12:2049, (1992).
Marshall, Science p. 1751, (1995).
Mastrangelo et al., Seminars in Oncology 23:4–21, (1996).
Sodroski et al., Nature 322:470–474, (1986).
Spencer et al. (1993) 262,1019–1024.
Eshar et al., "Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody–binding domains and the γ or ζ subunits of the immunoglobulin and T–cell receptors", *Proc. Natl. Acad. Sci., USA*, 90:720–724, (1993).
Kishimoto et al., "Cytokine Signal Transduction", *Cell*, 76:253–262, (1994).
Kolanus et al., "T Cell Activation by Clustered Tyrosine Kinases", *Cell*, 74:171–183, (1993).
Miyazaki et al., "Functional Activation of Jak1 and Jak3 by Selective Association with IL–2 Receptor Subunits", *Science*, 266:1045–1047, (1994).
Roberts et al., "Targeting of Human Immunodeficiency Virus–Infected Cells by CD28+ T Lymphocytes Armed With Universal T–Cell Receptors", *Blood*, 84(9):2878–2889, (1994).
Sato et al., "Multimeric cytokine receptors: common versus specific functions", *Current Opinion in Cell Biology*, 6:174–179, (1994).
Schlessinger et al., "Growth Factor Signaling by Receptor Tyrosine Kinases", *Neuron*, 9:383–391, (1992).
Stahl et al., "The Alphas, Betas, and Kinases of Cytokine Receptor Complexes", *Cell*, 74:587–590, (1993).
Ullrich et al., "Signal Transduction by Receptors with Tyrosine Kinase Activity", *Cell*,61:203–212, (1990).

(List continued on next page.)

Primary Examiner—Anthony C. Caputa
Assistant Examiner—Michael Pak
Attorney, Agent, or Firm—Sughrue, Mion, Zinn, Macpeak & Seas, PLLC

[57] **ABSTRACT**

Novel multispecific chimeric receptor DNA sequences, expression cassettes and vectors containing these sequences as well as cells containing the chimeric DNA and novel chimeric receptor proteins expressed from the sequences are provided in the present invention. The novel multispecific chimeric receptor DNA and amino acid sequences comprise at least three domains that do not naturally exist together: (1) a multispecific binding domain comprising at least two extracellular inducer-responsive clustering domains which serves to bind at least one specific inducer molecule, (2) a transmembrane domain, which crosses the plasma membrane, and (3) either a proliferation signaling domain that signals the cell to divide, or an effector function signaling domain which directs a host cell to perform its specialized function. Optionally, all the multispecific chimeric receptors may contain one or more intracellular inducer-responsive clustering domains attached to one or more of the cytoplasmic signaling domains or the transmembrane domain. The present invention also relates to novel hybrid multispecific chimeric receptors comprising at least one proliferation signaling domain and at least one effector function signaling domain together on the multispecific receptor molecule. The present invention further relates to therapeutic methods and strategies that employ the cells expressing these novel chimeric receptors for the treatment of cancer, infectious disease and autoimmune disease which may have greater therapeutic benefit over a combination of drug therapies.

47 Claims, 4 Drawing Sheets

OTHER PUBLICATIONS

Weiss et al., "T Cell Antigen Receptor Signal Transduction: A Tale of Tails and Cytoplasmic Protein–Tyrosine Kinases", *Cell*, 73:209–212, (1993).

Wilks et al., "Two Novel Protein–Tyrosine Kinases, Each with a Second Phosphotransferase–Related Catalytic Domain, Define a New Class of Protein Kinase", *Molecular and Cellular Biology*, 11(4):2057–2065, (1991).

Witthuhn et al., "JAK2 Associates with the Erythropoietin Receptor and Is Tyrosine Phosphorylated and Activated following Stimulation with Erythropoietin", *Cell*, 74:227–236, (1993).

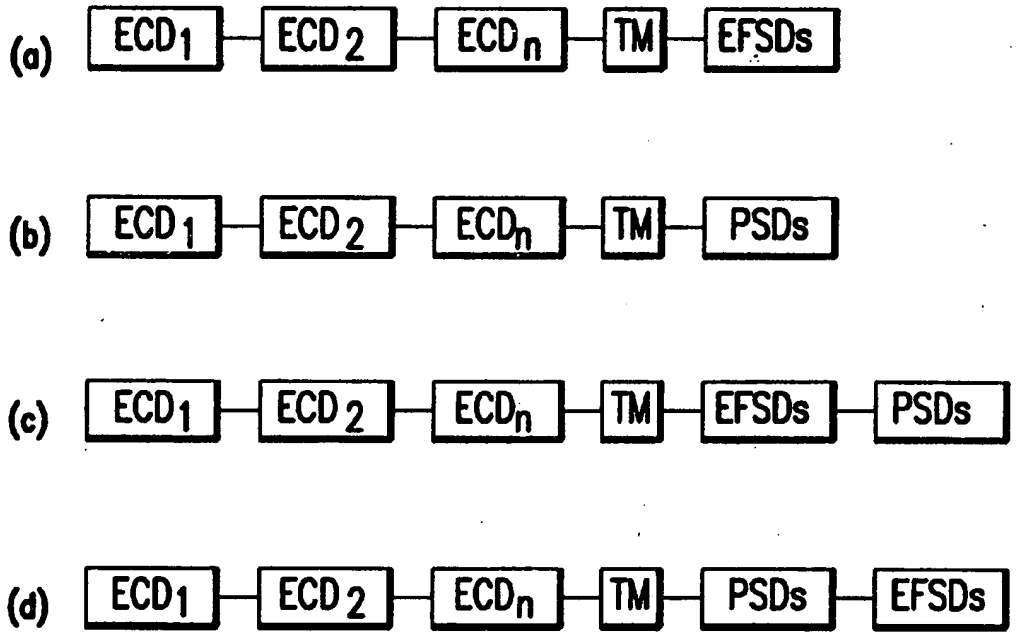
(a)
ECD1
ECD2
ECDn
TM
EFSDs
(b)
ECD1
ECD2
ECDn
TM
PSDs
(c)
ECD1
ECD2
ECDn
TM
EFSDs
PSDs
(d)
ECD1
ECD2
ECDn
TM
PSDs
EFSDs

FIG. 1

OLIGO 1 CTGCGTCAACACAGACTGTGAGGAGACGGTGACCAG

OLIGO 2 ACAGACTGTGAGGAGA

OLIGO 3 CTGCGTCAACACAGACTGACCCTTACCCTCAGAAGATTTA-
CCCGACCCCGAGGTCGACCCTGAGGAGACGGTGACCAG

OLIGO 4 AGAAGATTTACCCGAC

OLIGO 5 CTGCGTCAACACAGACTGACCGTCCTTCTTAGCGTCGTCC
TTCTTAGCGTCGTCCTTCTTAGCAGCGTCCTTCTTAGCGTCGT
CAGCGGAAGATGAGGAGACGGTGACCAG

OLIGO 6 GCGTCGTCCTTCTTAG

OLIGO 7 CTGCGTCAACACAGACTGTGGGGACGGTGGGGATGTGTG
AGTTTTGTCTGAGGAGACGGTGACCAG

OLIGO 8 CGGTGGGGATGTGTGA

OLIGO 9 CTGCGTCAACACAGACTGGTCCAGCTCCCCGTCCTGCGCTTC
GGCGCTCGATTCTTCCAGTTGCAGCTCTGAGGAGACGGTGAC
CAG

OLIGO 10 TCGGCGCTCGATTCTT

OLIGO 11 GCCCAGCACCACTTTCTTTGAGCTCACGGTGACCGT

OLIGO 12 ACTTTCTTTGAGCTCA

OLIGO 13 GCCCAGCACCACTTTCTTACCCTTACCCTCAGAAGATTTAC
CCGACCCCGAGGTCGACCCTGAGCTCACGGTGACCGT

OLIGO 14 GCCCAGCACCACTTTCTTACCGTCCTTCTTAGCGTCGTCCTT
CTTAGCGTCGTCCTTCTTAGCAGCGTCCTTCTTAGCGTCGTCA
GCGGAAGATGAGCTCACGGTGACCGT

OLIGO 15 GCCCAGCACCACTTTCTTTGGGGACGGTGGGGATGTGTGA
GTTTTGTCTGAGCTCACGGTGACCGT

FIG. 2A

OLIGO 16	GCCCAGCACCACTTTCTTGTCCAGCTCCCCGTCCTGCGCTTC GGCGCTCGATTCTTCCAGTTGCAGCTCTGAGCTCACGGTGAC CGT
OLIGO 17	TAGTCTAGGATCTACTGGCTGCAGTTCTTGCTCTCCTCTGTC
OLIGO 18	ACTGGCTGCAGTTCTT
OLIGO 19	AAAACTGTGCGTTACAATTCGTGGGTCCCCTCCTGAGGA
OLIGO 20	TACAATTCGTGGGTCC
OLIGO 21	TCCTATTGTAACAAATGCTTGCCCTGGTCCTCTCTGGAT
OLIGO 22	AAATGCTTGCCCTGGT

FIG. 2B

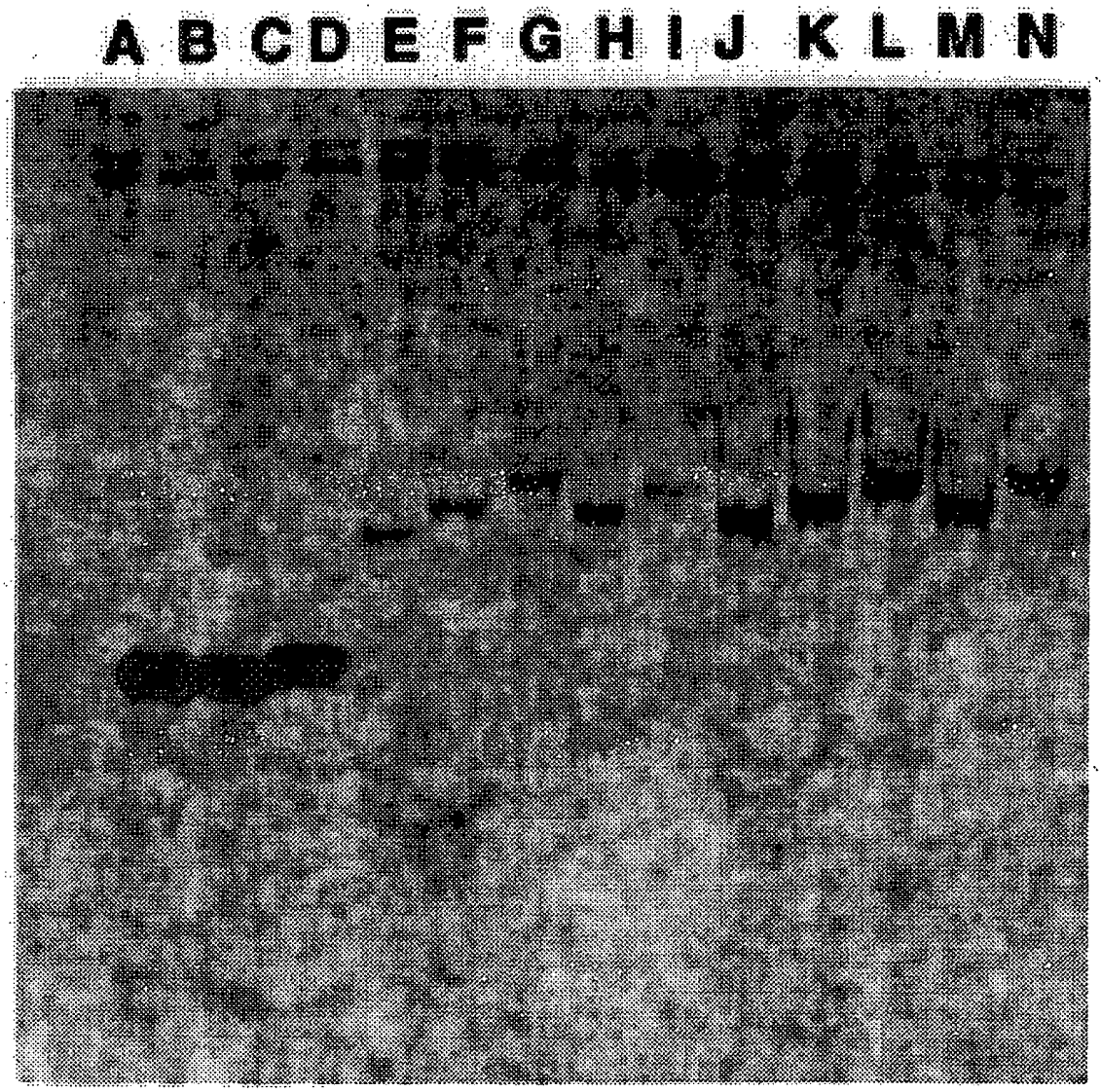

FIG.3

MULTISPECIFIC CHIMERIC RECEPTORS

This application is a continuation application of application Ser. No. 08/384,033, filed Feb. 5, 1995, which is now abandoned.

TECHNICAL FIELD

The field of the invention relates to the construction and use of novel multispecific chimeric receptors to overcome the obstacles presented by drug-resistance and genetic variation in infectious disease, cancer and autoimmune disease.

BACKGROUND

Agents designed to selectively inhibit the replication of a rapidly growing pathogen or cancer inevitably face a challenge from the development of drug-resistance. This problem is viewed by many clinicians as one of the major impediments to the effective management of malignant disease or the control of infectious agents which undergo major genetic variation. A related dilemma is presented by autoimmune disease, where a disease-causing cell population may be heterogeneous with respect to marker antigens which could be targeted as part of a therapeutic strategy.

The challenge of genetic variation to disease therapy is well illustrated by the problem of antiviral drug-resistance, though a similar situation holds true for resistance to antimicrobials and chemotherapeutics. The clinical emergence of drug-resistant virus has been documented in most instances in which antiviral therapy has been applied, including HIV, herpes simplex virus, varicella zoster virus, cytomegalovirus, influenza A virus, and rhinovirus (Richman, *Curr. Opin. Infect. Dis.* 3:819–823 (1990)). HIV infection provides a clear example of this problem, given its chronic, persistent nature, the high rate of viral replication, and the error-prone character of reverse transcriptase (RT). Resistance has been observed for every HIV antiviral tested, including nucleoside analogs (AZT, ddI, ddC, d4T and TSAO), non-nucleoside RT inhibitors (Merck's L-697,639 and Boehringer Ingelheim's nevirapine), and a protease inhibitor (Richman, *Annu. Rev. Pharmacol. Toxicol.* 32:149–164 (1993)).

The clinical significance of drug-resistance in HIV infection is best documented for AZT. While AZT reduced the rate of mortality in AIDS patients by over half during the first 12 months of treatment, ongoing therapy out to 24 months did not provide any additional advantage to the treated group. The apparent loss of AZT clinical efficacy correlates with the finding that by 12 months of therapy, approximately 90% of individuals with late-stage disease have developed AZT resistant virus (Richman et al., *J. AIDS* 3:743–746 (1990)). Similarly, the loss of antiviral activity observed with the non-nucleoside RT inhibitors within two months of their administration is associated with the rapid appearance of drug-resistant virus (Nunberg et al., *J. Virol.*; 65:4887–4892 (1991); Richman et al., *Proc. Natl. Acad. Sci.*, 88:11241–11245 (1991)).

Thus, while antiviral therapy with single agents can be quite effective for short periods, extended treatment of chronic or latent infections like HIV and the herpes viruses may require the application of combination therapies. Such a strategy is, however, often impractical due to the additional problems of cross-resistance as well as the cumulative side-effects of multiple agents. It is therefore desirable to design an therapeutic agent which can attack the pathogen at multiple points, in a fashion that minimizes cross-resistance, and has the safety profile of a single active agent. The present invention achieves this goal by providing multispecific chimeric receptors.

SUMMARY OF THE INVENTION

The present invention provides novel multispecific chimeric receptors and their applications to human disease therapy. The multispecific chimeric receptors of the present invention are single proteins possessing more than one antigen-binding and/or ligand-binding domain linked to an effector signaling domain and/or a proliferation signaling domain. A principal application of the novel multispecific chimeric receptors of the present invention is to combine the therapeutic benefits of two or more monospecific chimeric effector function receptors in a single protein for the treatment of a disease. In this manner, a multispecific protein product is provided which has the pharmacological profile of a single agent.

DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates the structures of the multispecific chimeric receptors discussed in the detailed description.

FIGS. 2A and 2B are listing of oligonucleotides (SEQ ID NOS: 1–22) as described in the Examples, infra.

FIG. 3 is an autoradiogram of immunoprecipitations of culture supernatants from 293 cells transfected with the following monospecific and multispecific antibody constructs as described in Examples 1 and 2: lane a, CD4-Fc; lane b, SAb(αgp41)-Fc; lane c, SAb(αgp120)-Fc; lane d, SAb(gp41)-SAb(αgp120)-Fc; lane e, SAb(αgp41)-L1-SAb (αgp120)-Fc; lane f, SAb(αgp41)-L2-SAb(αgp120)-Fc; lane g, SAb(αgp41)-L3-SAb(αgp120)-Fc; lane h, SAb (αgp41)-L4-SAb(αgp120)-Fc; lane i, SAb(αgp120)-CD4-Fc; lane j, SAb(αgp120)-L1-CD4-Fc; lane k, SAb(αgp120)-L2-CD4-Fc; lane 1, SAb(αgp120)-L3-CD4-Fc; lane m, SAb (αgp120)-L4-CD4-Fc.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As noted above, the present invention generally relates to novel multispecific chimeric receptors, namely, chimeric proliferation receptors, chimeric effector function receptors, and hybrids thereof, each comprising at least two ligand and/or antigen binding domains of different specifities, and DNA sequences encoding these novel chimeric receptors. Further aspects of the present invention will be discussed in detail below following a definition of terms employed herein.

Definitions:

The term "extracellular inducer-responsive clustering domain" or "ECD" refers to the portion of a protein of the present invention which is outside of the plasma membrane of a cell and binds to at least one extracellular inducer molecule as defined below. The ECD may include the entire extracytoplasmic portion of a transmembrane protein, a cell surface or membrane associated protein, a secreted protein, a cell surface targeting protein, a cell adhesion molecule, or a normally intracytoplasmic inducer-binding domain, and truncated or modified portions thereof. In addition, after binding one or more extracellular inducer molecule(s) defined below, the ECDs will become associated with each other by dimerization or oligomerization, i.e., "cluster".

The term "multispecific extracellular inducer-responsive clustering domain" or "MSECD" refers to more than one ECD, as defined above, linked in tandem, either covalently or non-covalently, on the same protein.

The term "intracellular inducer-responsive clustering domain" or "ICD" refers to the portion of a protein which is inside of the plasma membrane of a cell, that binds to at least one intracellular inducer molecule as defined below. After binding one or more intracellular inducer molecule(s), the ICDs will become associated with each other by dimerization or oligomerization, i.e., "cluster".

The term "transmembrane domain" or "TM" refers to the domain of the protein which crosses the plasma membrane and is derived from the inducer-binding ECD domain, the effector function signaling domain, the proliferation signaling domain or a domain associated with a totally different protein. Alternatively, the transmembrane domain may be an artificial hydrophobic amino acid sequence which spans the plasma cell membrane.

The term "proliferation signaling domain" or "PSD" refers to a protein domain which signals the cell to enter mitosis and begin cell growth. Examples include the human or mouse Janus kinases, including but not limited to, JAK1, JAK2, JAK3, Tyk2, Ptk-2, homologous members of the Janus kinase family from other mammalian or eukaryotic species, the IL-2 Receptor β and/or γ chains and other subunits from the cytokine receptor superfamily of proteins that may interact with the Janus kinase family of proteins to transduce a signal, and the cytoplasmic domains from the members of the superfamily of tyrosine kinase growth factor receptors, or portions, modifications or combinations thereof.

The term "effector function" refers to the specialized function of a differentiated cell. Effector function of a T cell, for example, may be cytolytic activity or helper activity including the secretion of cytokines.

The term "effector function signaling domain" or "EFSD" refers to the portion of a protein which transduces the effector function signal and directs the cell to perform its specialized function. While usually the entire EFSD will be employed, in many cases it will not be necessary to use the entire chain. To the extent that a truncated portion of the EFSD may find use, such truncated portion may be used in place of the intact chain as long as it still transduces the effector function signal. Examples are the ζ chain of the T cell receptor or any of its homologs (e.g., η chain, FcεR1-γ and -β chains, MB1 (Igα) chain, B29 (Igβ) chain, etc.), CD3 polypeptides (Δ, δ and ε), syk family tyrosine kinases (Syk, ZAP 70, etc.), src family tyrosine kinases (Lck, Fyn, Lyn, etc.) and other molecules involved in T cell transduction, such as CD2, CD5 and CD28.

The term "linker" or "linker region" refers to an oligo- or polypeptide region of from about 1 to 100 amino acids that links together any of the above described domains of the MSCRs defined above. The amino acid sequence is typically derived from a protein other than the ICDs, ECDs, EFSDs, PSDs or TM domains. Examples of linker regions are linker 212 and linker 205 as referenced in Bedzyk et al., *J. Biol. Chem.*, 265:18615–18620 (1990) and Gruber et al., *J. Immunol.*, 152:5368–5374 (1994), respectively.

The term "membrane hinge" refers to a hydrophilic polypeptide sequence found in membrane-bound immunoglobulin heavy chains, where it is attached to the extracellular side of the TM domain (Bensmana & Lefranc, *Immunogenetics*, 32:321–330 (1990)). As used in the present invention, the membrane hinge may be considered a subset of linkers.

The term "chimeric inducer-responsive proliferation receptor" or "CPR" refers to a chimeric receptor that comprises an extracellular inducer responsive clustering domain (ECD), a transmembrane domain (TM) and at least one proliferation signaling domain (PSD). The ECD and PSD are not naturally found together on a single protein receptor. Optionally, this chimeric receptor may also contain an effector function signaling domain to form a "hybrid MSCR" as defined below.

The term "chimeric effector function receptor" or "CEFR" refers to a chimeric receptor that comprises an extracellular domain, transmembrane domain, and at least one cytoplasmic domain as described in U.S. Pat. No. 5,359,046 or at least one EFSD domain as described above. The extracellular domain serves to bind to an extracellular inducer and transmit a signal to the cytoplasmic domain which transduces an effector function signal to the cell.

The term "multispecific chimeric effector function receptor" or "MSCEFR" refers to a chimeric effector function receptor which contains a multispecific extracellular inducer-responsive clustering domain (MSECD).

The term "multispecific chimeric proliferation receptor" or "MSCPR" refers to a chimeric inducer-responsive proliferation receptor which contains a multispecific extracellular inducer-responsive clustering domain (MSECD).

The term "hybrid MSCR" refers to a chimeric receptor that comprises a MSECD, a TM and at least one EFSD and at least one PSD linked together, in either order, directly or via a linker region to the transmembrane domain in either order.

The term "multispecific chimeric receptor" or "MSCR" refers to a chimeric receptor that comprises a multispecific ECD (MSECD), a transmembrane domain (TM) and at least one effector function signaling domain (EFSD) and/or at least one proliferation signaling domain (PSD (i.e. MSCEFRS, MSCPRs and hybrid MSCRs)). In addition, the MSCEFRS, MSCPRs and hybrid MSCRs of the present invention may also have one or more ICDs attached to one or more of their cytoplasmic domains.

The term "extracellular inducer molecule" refers to a ligand or antigen which binds to an ECD and induces the clustering of the ECD or MSECD as described above, or portions or modifications of the extracellular inducer molecule that are still capable of binding to the ECD and inducing the clustering of an MSECD. To facilitate clustering, the extracellular inducer molecule may be intrinsically bivalent or multivalent; or it may be presented to the ECD in a bivalent or multivalent form, eg., on the surface of a cell or a virus.

The term "intracellular inducer molecule" refers to a natural or synthetic ligand that can be delivered to the cytoplasm of a cell, and binds to and induces the clustering of an intracellular-inducer responsive domain (ICD). To facilitate clustering, the intracellular inducer molecule may be intrinsically bivalent or multivalent.

The term "multispecific antibody" refers to an antibody molecule, or truncations or modifications thereof, that comprises two or more ECDs of different specificities.

The term "modifications" refers to an addition of one or more amino acids to either or both of the C- and N-terminal ends of the extracellular or intracellular inducer molecules (in the case where these are proteins) or, the ECDs, PSDs, EFSDS, ICDs or TMs, a substitution of one or more amino acids at one or more sites throughout these proteins, a deletion of one or more amino acids within or at either or both ends of these proteins, or an insertion of one or more amino acids at one or more sites in these proteins such that the extracellular or intracellular inducer molecule binding to the ECD or ICD is retained or improved as measured by

binding assays known in the art, for example, Scatchard plots, or such that the PSD, EFSD, ICD or TM domain activities are retained or improved as measured by one or more of the proliferation assays or effector assays described below. In addition, modifications can be made to the extracellular or intracellular inducer molecules (where they are proteins) and to the corresponding ECDs or ICDs to create an improved receptor-ligand binding pair.

The term "variant" refers to a DNA fragment encoding an extracellular or intracellular inducer molecule, or an ECD, PSD, EFSD, ICD or TM domain that may further contain an addition of one or more nucleotides internally or at the 5' or 3' end of the DNA fragment, a deletion of one or more nucleotides internally or at the 5' or 3' end of the DNA fragment or a substitution of one or more nucleotides internallly or at the 5' or 3' end of the DNA fragment such that the extracellular or intracellular inducer molecule binding to the ECD or ICD is retained or improved as measured by binding assays known in the art, for example, Scatchard plots, or such that the PSD, EFSD, ICD or TM domain activities are retained or improved as measured by one or more of the proliferation assays or effector assays described below. In addition, variants of the DNA sequences encoding the extracellular and intracellular inducer molecules (where they are proteins) and the corresponding ECDs and ICDs can be made so as to create an improved receptor-ligand binding pair.

In a general embodiment, the present invention relates to novel multispecific chimeric receptors, nucleic acid sequences encoding the receptors, the vectors containing the nucleic acids encoding the receptors, the host cells expressing these novel multispecific chimeric receptors, and methods of using these novel multispecific chimeric receptors as therapeutics. Three types of multispecific chimeric receptors (MSCR) are provided herein, namely, multispecific chimeric proliferation receptors (MSCPR) (FIG. 1(*b*)), multispecific chimeric effector function receptors (MSCEFR) (FIG. 1(*a*)) and hybrid MSCRs comprising both an effector signaling domain and a proliferation signaling domain (FIGS. 1(*c*) and 1(*d*)). In each category of receptors, the multispecific binding domain, the effector function signaling domain, and the proliferation signaling domain do not naturally exist together on a single protein.

In one particular embodiment, the present invention relates to a multispecific chimeric proliferation receptor (MSCPR) designed to be expressed in cells, which in turn proliferate in response to at least one specific extracellular inducer molecule. The three domains which comprise a MSCPR are: (1) a multispecific binding domain comprising at least two extracellular inducer-responsive clustering domains (ECDs) which serves to bind to at least one specific extracellular molecule, (2) a transmembrane domain, which crosses the plasma membrane and, (3) at least one proliferation signaling domain that signals the cell to divide (FIG. 1(*b*)).

The cells bearing the MSCPRs of the present invention will expand in number in response to the binding of one or more different specific extracellular molecules to an extracellular inducer-responsive clustering domain of the MSCPR. In each instance, the extracellular inducer molecule binds to at least one ECD, which results in the dimerization or oligomerization of the MSECDs to each other. The clustering of these MSECDs signals activation of the proliferation domain(s).

In another embodiment, the present invention relates to a novel multispecific chimeric effector function receptor (MSCEFR) designed to be expressed in cells, which when activated by the binding of at least one specific extracellular inducer molecule, will induce a specialized effector function of a differentiated cell. The three domains that comprise a MSCEFR are: (1) a multispecific binding domain comprising at least two extracellular inducer-responsive clustering domains (ECDs) which serves to bind to at least one specific extracellular inducer molecule, (2) a transmembrane domain, which crosses the plasma membrane and, (3) at least one effector function signaling domain which tranduces the effector function signal and directs the cell to perform its specialized function (FIG. 1(*a*)).

The cells bearing the MSCEFRs of the present invention will express effector function in response to the binding of one or more different specific extracellular inducer molecules to an extracellular inducer-responsive clustering domain of the MSCEFR. In each instance, the extracellular inducer molecule binds to at least one ECD, which results in the dimerization or oligomerization of the MSECDs to each other. The clustering of these MSECDs signals activation of the effector function signaling domain(s).

In yet another embodiment, the present invention relates to a novel hybrid multispecific chimeric receptor (hybrid MSCR) containing a proliferation signaling domain and an effector function signaling domain together on the same multispecific receptor. In this particular embodiment, the hybrid receptor comprises a MSECD and TM described above, and additionally comprises at least one effector function signaling domain and at least one proliferation signaling domain joined together on the same protein (FIGS. 1(*c*) and 1(*d*)). Thus, the multispecific extracellular inducer responsive clustering domains (MSECDs) of the hybrid MSCR are linked via a transmembrane domain to two different types of signal transducing domains. Either the proliferation signaling domain or the effector function signaling domain may be linked to the transmembrane domain which is further linked on its 3' end to the second signaling domain either directly or through a linker region. It is contemplated that the preparation of this novel hybrid MSCR will activate proliferation and effector function simultaneously in a host cell upon the binding of at least one extracellular inducer molecule to one or more the ECDs of the hybrid MSCR of the present invention.

In yet another aspect of the present invention, a novel multispecific chimeric proliferation receptor containing a multispecific extracellular inducer-responsive clustering domain (MSECD), and a proliferation signaling domain (PSD) is provided together in the same receptor protein with an intracellular inducer-responsive clustering domain (ICD). In this embodiment, a receptor is constructed as one protein comprising in the N-terminal to C-terminal direction a multispecific ECD, TM domain, an ICD and a PSD. Alternatively, a receptor may be constructed as one protein comprising in the N-terminal to C-terminal direction an MSECD, TM domain, PSD and an ICD. In preparing the multispecific chimeric inducer binding receptors of the present embodiment, one may separate one or more domains of each receptor with a linker or membrane hinge region. Additionally, more than one ICD and PSD may be attached directly or via a linker or membrane hinge region to each other to form multiple ICDs and PSDS. Upon introduction of these novel inducer-binding chimeric proliferation receptors into a host cell, one may modulate proliferation of the cell by either an extracellular inducer, an intracellular inducer or a combination of these two different inducer molecules. The embodiment of this aspect of the invention may be modified even further by attaching an effector

function signaling domain (EFSD) at the N-terminal or C-terminal end of the PSD or the ICD. A MSECD and multiple ICDs and/or PSDs may be employed in the construction of these receptors. Upon expression of these novel hybrid multispecific receptors containing both an MSECD and an ICD in a host cell, one may modulate proliferation and effector signaling by adding either an extracellular inducer, an intracellular inducer or a combination of these two different inducer molecules.

The extracellular inducer-responsive clustering domain (ECD) may be obtained from any of the wide variety of extracellular domains of eukaryotic transmembrane proteins, secreted proteins or other proteins associated with ligand binding and/or signal transduction. The ECD may be part of a protein which is monomeric, homodimeric, heterodimeric, or associated with a larger number of proteins in a non-covalent or disulfide-bonded complex. To create the multispecific binding domains of the present invention, two or more individual binding domains are connected to each other on a single protein, either covalently or noncovalently. An oligo- or polypeptide linker region may be used to connect these domains to each other.

In particular, the ECDs may consist of monomeric, dimeric or tetrameric immunoglobulin molecules, or portions or modifications thereof, which are prepared in the following manner.

The full-length IgG heavy chain comprising the VH, CH1, hinge, and the CH2 and CH3 (Fc) Ig domains may be fused to a EFSD, PSD or ICD of an MSCR via the appropriate transmembrane domain. If the VH domain alone is sufficient to confer antigen-specificity (so-called "single-domain antibodies"), homodimer formation of the MSCR is expected to be functionally bivalent with regard to antigen binding sites. If both the VH domain and the VL domain are necessary to generate a fully active antigen-binding site, both the VH-containing MSCR and the full-length IgL chain are introduced into cells to generate an active antigen-binding site. Dimer formation resulting from the intermolecular Fc/hinge disulfide bonds results in the assembly of MSCRs with extracellular domains resembling those of IgG antibodies. Derivatives of these MSCRs include those in which only non-Fc regions of the heavy chain are employed in the fusion. For example, the VH domain (and the CH1 domain) of the heavy chain can be retained in the extracellular domain of the MSCR, but MSCR dimers are not formed. As above, the full-length IgL chain can be introduced into cells to generate an active antigen-binding site.

As indicated, the ECD may consist of an Ig heavy chain which may in turn be covalently associated with Ig light chain by virtue of the presence of the CH1 region, or may become covalently associated with other Ig heavy/light chain complexes by virtue of the presence of hinge, CH2 and CH3 domains. The two heavy/light chain complexes may have different specificities, thus creating a MSCR which binds two distinct antigens. Depending on the function of the antibody, the desired structure and the signal transduction, the entire chain may be used or a truncated chain may be used, where all or a part of the CH1, CH2, or CH3 domains may be removed or all or part of the hinge region may be removed.

Because association of both the heavy and light V domains are required to generate a functional antigen binding site of high affinity, in order to generate an antibody-containing MSCR with the potential to bind antigen, a total of two molecules will typically need to be introduced into the host cell. Therefore, an alternative and preferred strategy is to introduce a single molecule bearing a functional antigen binding site. This avoids the technical difficulties that may attend the introduction and coordinated expression of more than one gene construct into host cells. This "single-chain antibody" (SAb) is created by fusing together the variable domains of the heavy and light chains using an oligo- or polypeptide linker, thereby reconstituting an antigen binding site on a single molecule.

Single-chain antibody variable fragments (SAbFv) in which the C-terminus of one variable domain (VH or VL) is tethered to the N-terminus of the other (VL or VH, respectively), via a oligo- or polypeptide linker, have been developed without significantly disrupting antigen binding or specificity of the binding (Bedzyk e al. (1990) *J. Biol. Chem.*, 265:18615; Chaudhary et al. (1990) *Proc. Natl. Acad. Sci.*, 87:9491). The SAbFvs used in the present invention may be of two types depending on the relative order of the VH and VL domains: VH-l-VL or VL-i-VH (where "1" represents the linker). These SAbFvs lack the constant regions (Fc) present in the heavy and light chains of the native antibody. In another aspect of the present invention, the SAbFv fragment may be fused to all or a portion of the constant domains of the heavy chain, and the resulting ECD is joined to the EFSD, PSD or ICD via an appropriate transmembrane domain that will permit expression in the host cell. The resulting MSCRs differ from the SAbFvs, described above, in that upon binding of antigen they initiate signal transduction via their cytoplasmic domain.

Single-chain derivatives of T cell receptors (SCTCRs) in which the variable regions of the T cell receptor α and β chains are joined together by an appropriate oligo- or polypeptide linker may also be employed as one or more of the ECDs contained within an MSCR.

To aid in the proper folding and efficient expression of the MSCRs, including MSCEFRs, MSCPRs and hybrid MSCRS, the antibody-derived ECDs may be connected at their C-terminal end to one of a number of membrane hinge regions which are a normal part of membrane-bound immunoglobulin molecules. For example, the eighteen amino acids of the IGHG3 M1 exon may be used (Bensmana and Lefranc, *Immunogenet.*, 32:321–330 (1990)). The TM domain is attached to the C-terminal end of the membrane hinge. It is also contemplated that membrane hinge sequences may be used to connect non-antibody derived ECDs to the transmembrane domains to increase CPR expression.

Diabodies may also be used as ECDs in the present invention. Diabodies contain two chimeric immunoglobulin chains, one of which comprises a VH domain connected to a VL domain on the same polypeptide chain (VH-VL). A linker that is too short to allow pairing of the VH and VL domains on this chain with each other is used so that the domains will pair with the complementary VH and VL domains on the other chimeric immunoglobulin chain to create two antigen-binding sites (Holliger et al., *Proc. Natl. Acad. Sci.* 90:6444–6448 (1993)). As described above, one of these chains is linked to the membrane hinge and/or the TM domain, which in turn is linked to the EFSD, PSD and/or ICD. The other chain (not connected covalently to a EFSD, PSD or ICD) will be co-expressed in the same cell to create a MSCR with a diabody ECD which will respond to two different extracellular inducer molecules.

In the present invention, the SCFv fragment or the single domain antibody may be fused to all or a portion of the constant domains of the heavy chain or the light chain before

being linked to each other to form the multispecific ECD. The MSECDs used in the present invention may comprise (N- to C-terminal) a SCFv fragment linked to another SCFv domain that in turn is linked to all or part of the constant domains (CH1, hinge, CH2 and CH3).

To aid in the proper folding and efficient expression of the MSCRs of the present invention, the antibody-derived ECDs may be connected at their C-terminal end to one of a number of "membrane hinge regions" which are a normal part of membrane-bound immunoglobulin molecules. The TM domain is attached to the C-terminal end of the membrane hinge. For example, eighteen amino acids from the N-terminal end of the IGHG3 M1 exon may be used (Bensmana and Lefranc, *Immunogenet.,* 32:321–330 (1990)). It is contemplated that membrane hinge sequences may be used to connect non-antibody derived ECDs to the transmembrane domains to increase CPR and CEFR expression. In the present invention, the membrane hinge region may also be employed like other linker regions, eg., be attached on either side of a TM, PSD, or ECD.

Ligand-binding domains from naturally occurring receptors may also be used as ECDs to prepare the MSECDs of the present invention, where the receptors are surface membrane proteins, including cell differentiation antigens such as CD4 and CD8, cytokine or hormone receptors or cell adhesion molecules such as ICAM or LFA-1. The receptor may be responsive to a natural ligand, an antibody or fragment thereof, a synthetic molecule, e.g., drug, or any other agent which is capable of inducing a signal. In addition, either member of a ligand/receptor pair, where one is expressed on a target cell such as a cancer cell, a virally infected cell or an autoimmune disease causing cell, may also be used as an ECD in the present invention. In addition, the receptor-binding domains of soluble ligands or portions thereof could be employed as ECDs in the MSECDs of the present invention. In addition, binding portions of antibodies, cytokines, hormones, or serum proteins can be used. Furthermore, secreted targeting molecules such as IL-14 or the soluble components of the cytokine receptors such as IL-6R, IL-4R, and IL-7R can be used as ECDs (Boulay and Paul *Current Biology* 3: 573–581, 1993).

Where a receptor is a molecular complex of proteins, where only one chain has the major role of binding to the ligand, it will usually be desirable to use solely the extracellular portion of the ligand binding protein. Where the extracellular portion may complex with other extracellular portions of other proteins or form covalent bonding through disulfide linkages, one may also provide for the formation of such dimeric or multimeric extracellular regions. Also, where the entire extracellular region is not required, truncated portions thereof may be employed, where such truncated portion is functional. In particular, when the extracellular region of CD4 is employed, one may use only those sequences required for binding of gp120, the HIV envelope glycoprotein. In the case in which immunoglobulin-derived sequences are used to create a multispecific ECD, one may simply use the antigen binding regions of the antibody molecule and dispense with the constant regions of the molecule (for example, the Fc region consisting of the CH2 and CH3 domains).

To create the multispecific ECDs of the present invention, two or more individual ECDs are connected to each other, either covalently or noncovalently, on a single protein molecule. An oligo- or polypeptide linker, an Fc hinge or membrane hinge region may be used to connect these domains to each other. The MSECDs of the present invention may comprise two or more of the different ECDs described above connected together in different combinations. For example, two or more ECDs containing immunoglobulin sequences, (e.g. SCFvs and/or single-domain antibodies) may be linked to each other. In another example, two or more ECDs from membrane proteins (e.g. cytokine receptors and/or CD antigens) may be linked to each other. In yet another example, a MSECD may consist of a mixture of ligand-binding domains and immunoglobulin-derived domains eg., an ECD from CD4 may be joined to a SCFv.

The proliferation signaling domains (PSDS) that comprise the MSCPRs and hybrid MSCRs of the present invention may be obtained from the cytoplasmic signal-transducing domains of the cytokine/hematopoietin receptor superfamily. The members of this mammalian receptor superfamily can transduce proliferative signals in a wide variety of cell types. The cytoplasmic domains of the signal-transducing subunits may contain conserved motifs that are critical for the transduction of proliferative signals (Bazan, *Current Biology,* 3:603–606 (1993); Boulay and Paul, *Current Biology,* 3:573–581 (1993); Wells, *Current Opinion in Cell Biology,* 6:163–173 (1994); Sato and Miyajima, *Current Opinion in Cell Biology,* 6:174–179 (1994); Stahl and Yancopoulos, *Cell,* 74:587–590 (1993); Minami et al., *Ann. Rev. Immunol.,* 11:245–267 (1993); Kishimoto et al., *Cell,* 76:253–262 (1994)). The signal-transducing components of these cytokine receptors to be used as PSDs in the present invention include, but are not limited to, Interleukin-2 receptor β (IL-2Rβ), IL-2Rγ, IL-3Rβ, IL-4R, IL-5Rα, IL-5Rβ, IL-6R, IL-6R gp130, IL-7R, IL-9R, IL-12R, IL-13R, IL-15R, EPO-R (erythropoietin receptor), G-CSFR (granulocyte colony stimulating factor receptor), GM-CSFRα (granulocyte macrophage colony stimulating factor receptor α), GM-CSFRβ, LIFRα (leukemia inhibitory factor receptor α), GHR (growth hormone receptor), PRLR (prolactin receptor), CNTFR (ciliary neurotrophic factor receptor), OSMR (oncostatin M receptor) IFNRα/β (interferon α/β receptor), IFNRγ, TFR (tissue factor receptor), and TPOR (thrombopoietin or mpl-ligand receptor)(Minami et al., *J. Immunol.,* 152:5680–5690 (1994); Boulay and Paul, *Current Biology,* 3:573–581 (1993); Wells, *Current Opinion in Cell Biology,* 6:163–173 (1994)).

The proliferation signaling domains (PSDs) that comprise the MSCPRs and hybrid MSCRs of the present invention may be obtained from the signal-transducing domains of the tyrosine kinase growth factor receptor superfamily or from oncogenes or proto-oncogenes which are related to this growth factor family (Schlessinger and Ullrich, *Cell,* 61:203–212 (1990), Ullrich and Schlessinger, *Neuron,* 9:383–391 (1992)). The members of this mammalian receptor superfamily can transduce proliferative signals in a wide variety of cell types. The cytoplasmic domains of the signal-transducing subunits contain tyrosine kinase domains that are critical for the transduction of proliferative signals. The growth factor receptors, proto-oncogenes, and oncogenes to be used as PSDs in the present invention include, but are not limited to epidermal growth factor receptor (EGF-R), HER2/neu, HER3/c-erbB-3, Xmrk, Insulin-R, IGF-1-R (insulin-like growth factor-1 receptor), IRR, PDGF-R-A (platelet-derived growth factor receptor-A), PDGF-R-B (platelet-derived growth factor receptor-B), CSF-1-R (colony-stimulating factor-1 receptor), c-kit, FGF-R (fibroblast growth factor receptor), acidic FGF-R, and basic FGF-R (Ullrich and Schlessinger, *Cell,* 61:203–212 (1990)).

The proliferation signaling domains employed in constructing the MSCPRs and hybrid MSCRs of the present

invention may also be obtained from any member of the Janus or JAK eukaryotic family of tyrosine kinases, including Tyk2, JAKI, JAK2, JAK3 and Ptk-2. Members of the Janus kinase family are found in all cell types. They associate with various signal transducing components of the cytokine receptor superfamily discussed above and respond to the binding of extracellular inducer by the phosphorylation of tyrosines on cytoplasmic substrates (Stahl and Yancopoulos, *Cell,* 74:587–590 (1993)). They are thus an integral part of the control of cell proliferation in many different kinds of cells. The members of this family are marked by similar multidomain structures and a high degree of sequence conservation. Unique among tyrosine kinases, the Janus kinase family may have two non-identical tandem kinase-like domains, only one of which may have catalytic activity (Firmbach-Kraft et al., Oncogene, 5:1329–1336 (1990); Wilks et al., *Mol. Cell. Biol.,* 11:2057–2065 (1991); Harpur et al., *Oncogene,* 7:1347–1353 (1992)). The kinase activity of the Janus kinases is usually activated after the binding of ligands to their associated cytokine family receptors and the oligomerization of the receptors (Stahl and Yancopoulos, *Cell,* 74:587–590 (1993)). This activation, in turn, triggers the initiation of intracellular signaling cascades (Witthuhn et al., *Nature,* 370:153–157 (1994); Russell et al., *Science,* 366:1042–1044 (1994); Kawamura et al., *Proc. Natl. Acad. Sci.,* 91:6374–6378 (1994); Miyazaki et al., *Science,* 266:1045–1047 (1994); Johnston et al., *Nature,* 370:151–153 (1994); Asao et al., *FEBS Letters,* 351:201–206 (1994)).

The effector function signaling domains (EFSDs) employed in the MSCEFRs and hybrid MSCRs of the present invention may be derived from a protein which is known to activate various second messenger pathways. One pathway of interest is that involving phosphatidylinositol-specific phospholipase hydrolysis of phosphatidylinositol-4, 5-biphosphate, and production of inositol-1,4,5-trisphosphate and diacylglycerol. The calcium mediated pathway, the tyrosine and serine/threonine kinase and phosphatase pathway, the adenylate cyclase, and the guanylate cyclase pathways may also be second messenger pathways. EFSDs of interest include proteins with ARAM motifs (Reth, *Nature,* 338:383–384 (1989); Weiss, *Cell,* 73:209–212 (1993)), for example, the ζ chain of the T-cell receptor, the η chain, which differs from the ζ chain only in its most C-terminal exon as a result of alternative splicing of the ζ mRNA, the γ and β subunit of the FcεR1 receptor, the MB1 (Igα) and B29 (Igβ) chains of the B cell receptor, and the δ, γ, and ε chains of the T-cell receptor (CD3 chains), other proteins homologous to the above protein subunits including synthetic polypeptides with ARAM motifs, and such other cytoplasmic regions which are capable of transmitting a signal as a result of interacting with other proteins capable of binding to an inducer (Romeo et al., *Cell,* 68:889–897 (1992); Weiss, *Cell,* 73:209–212 (1993)). The syk family of tyrosine kinases may also be used as effector function signaling domains in the present invention. The clustering of these domains from Syk and ZAP-70 leads to the activation of T cell cytolytic activity (Kolanus et al., *Cell,* 74:171–183 (1993)). In addition, the src family of tyrosine kinases (Lck, Fyn, Lyn, etc.) (Rudd et al., *Immunology Today,* 15:225–234 (1994)) and molecules such as CD2, CD5 and CD28, which are involved in T cell transduction, may also be used as EFSDs in the present invention. A number of EFSDs or functional fragments or mutants thereof may be employed, generally ranging from about 50 to 1500 amino acids each, where the entire naturally occurring cytoplasmic region may be employed or only an active portion thereof.

The intracellular clustering domain (ICD) can be obtained from the inducer binding domains of a variety of intracellular proteins. For example, eukaryotic steroid receptor molecules can be used as ICDs (e.g. the receptors for estrogen, progesterone, androgens, glucocorticoids, thyroid hormone, vitamin D, retinoic acid, 9-cis retinoic acid and ecdysone). In addition, variants of steroid and other receptors which fail to bind their native inducer, but still bind to an antagonist, can be prepared by one skilled in the art and used to make the CPRs of this invention. For example, a C-terminal deletion mutant of the human progesterone receptor, which fails to bind progesterone, can be clustered by the addition of progesterone antagonists, including RU 486 (Wang et al., *Proc Natl Acad Sci* 91: 8180–8184, 1994). Binding domains from the eukaryotic immunophilin family of molecules may also be used as ICDs. Examples include but are not limited to members of the cyclophilin family: mammalian cyclophilin A, B and C, yeast cyclophilins 1 and 2, Drosophila cyclophilin analogs such as ninaA; and members of the FKPB family: the various mammalian isoforms of FKBP and the FKBP analog from Neurospora (Schreiber, *Science,* 251:283–287 (1991), McKeon, *Cell,* 66:823–826, (1991), Friedman and Weissman, *Cell,* 66:799–806, (1991), Liu et al., *Cell,* 66:807–815 (1991)). For example, the inducer binding portion of the immunophilin, FKBP12, which can be clustered in the cytoplasm by the addition of FK1012, a synthetic dimeric form of the immunosuppressant FK506 (Spencer et al., *Science* 262: 1019–1024 (1993) can be used as an ICD.

The transmembrane domain may be contributed by the protein contributing the multispecific extracellular inducer clustering domain, the protein contributing the effector function signaling domain, the protein contributing the proliferation signaling portion, or by a totally different protein. For the most part it will be convenient to have the transmembrane domain naturally associated with one of the domains. In some cases it will be desirable to employ the transmembrane domain of the ζ, η or FcεR1γ chains which contain a cysteine residue capable of disulfide bonding, so that the resulting chimeric protein will be able to form disulfide linked dimers with itself, or with unmodified versions of the ζ, η or FcεR1γ chains or related proteins. In some instances, the transmembrane domain will be selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex. In other cases it will be desirable to employ the transmembrane domain of ζ, η, FcεR1-γ and -β, MB1 (Igα), B29 or CD3-γ, ζ, or ε, in order to retain physical association with other members of the receptor complex.

Extracellular inducer molecules employed in the present invention can be antigens which bind the immunoglobulin-derived ECDs, described above. These may include viral proteins, (e.g. gp120 and gp41 envelope proteins of HIV, envelope proteins from the Hepatitis B and C viruses, the gB and other envelope glycoproteins of human cytomegalovirus, the envelope proteins from the Kaposi's sarcoma-associated herpesvirus), and surface proteins found on cancer cells in a specific or amplified fashion, (e.g. the IL-14 receptor, CD 19 and CD 20 for B cell lymphoma, the Lewis Y and CEA antigens for a variety of carcinomas, the Tag72 antigen for breast and colorectal cancer, EGF-R for lung cancer, and the HER-2 protein which is often amplified in human breast and ovarian carcinomas). For other receptors, the receptors and ligands of particular interest are CD4, where the ligand is the HIV gp120 envelope

glycoprotein, and other viral receptors, for example ICAM, which is the receptor for the human rhinovirus, and the related receptor molecule for poliovirus.

The intracellular inducer molecules employed in the present invention must be molecules which can be delivered to the cytoplasm. For example, the inducer may be lipophilic, or be transported into the cell by active transport or pinocytosis, by fusion with a liposome carrying the inducer, or by semi-permeabilization of the cell membrane. The intracellular inducers cluster the ICDs which make up the CIPRs of the present invention. Examples of inducers include, but are not limited to synthetic dimeric molecules such as FK1012 (Spencer et al., *Science,* 262:1019–1024 (1993)) or dimeric derivatives of the binding domains of other immunophilin binding molecules such as cyclosporin, rapamycin and 506BD (Schreiber, *Science,* 251:283–287 (1991), McKeon, *Cell,* 66:823–826, (1991)). Steroids, such as estrogen, progesterone, the androgens, glucocorticoids, thyroid hormone, vitamin D, retinoic acid, 9-cis retinoic acid or ecdysone, or antagonists or derivatives of these molecules may also be used as intracellular inducer molecules. In particular, the steroid antagonist RU 486 may be used (Wang et al., *Proc. Natl. Acad. Sci.,* 91:8180–8184 (1994)).

In some instances, a few amino acids at the joining region of the natural protein domain may be deleted (eg., truncated), usually not more than 30, more usually not more than 20. Also, one may wish to introduce a small number of amino acids at the borders, usually not more than 30, more usually not more than 20 (linkers or the membrane hinge region). The deletion or insertion of amino acids will usually be as a result of the needs of the construction, providing for convenient restriction sites, ease of manipulation, improvement in levels of expression, proper folding of the molecule or the like. In addition, one may wish to substitute one or more amino acids with a different amino acid (i.e., a modification) for similar reasons, usually not substituting more than about five amino acids in any one domain. The PSDs and EFSDs will generally be from about 50 to 1500 amino acids, depending upon the particular domain employed, while the transmembrane domain will generally have from about 20 to 35 amino acids. The individual ECDs will generally be from about 50 to 1500 amino acids, depending on the particular domain employed. The MSECDs will usually contain between two and twenty ECDs, more preferably between two and ten ECDs, and most preferably between two and five ECDs.

Normally, the signal sequence at the 5' terminus of the open reading frame (ORF) which directs the chimeric protein to the surface membrane will be the signal sequence of the ECD. However, in some instances, one may wish to exchange this sequence for a different signal sequence. However, since the signal sequence will be removed from the protein during processing, the particular signal sequence will normally not be critical to the subject invention.

The chimeric construct, which encodes the chimeric protein according to this invention will be prepared in conventional ways. Since, for the most part, natural sequences may be employed, the natural genes may be isolated and manipulated, as appropriate, so as to allow for the proper joining of the various domains. Thus, one may prepare the truncated portion of the sequence by employing the polymerase chain reaction (PCR), using appropriate primers which result in deletion of the undesired portions of the gene. Alternatively, one may use primer repair, where the sequence of interest may be cloned in an appropriate host. In either case, primers may be employed which result in termini, which allow for annealing of the sequences to result in the desired open reading frame encoding the chimeric protein. Thus, the sequences may be selected to provide for restriction sites which are blunt-ended, or have complementary overlaps.

If desired, the multispecific extracellular domain may also include the transcriptional initiation region, which will allow for expression in the target host. Alternatively, one may wish to provide for a different transcriptional initiation region, which may allow for constitutive or inducible expression, depending upon the target host, the purpose for the introduction of the subject chimeric protein into such host, the level of expression desired, the nature of the target host, and the like. Thus, one may provide for expression upon differentiation or maturation of the target host, activation of the target host, or the like.

A wide variety of promoters have been described in the literature, which are constitutive or inducible, where induction may be associated with a specific cell type or a specific level of expression. Alternatively, a number of viral promoters are known which may also find use. Promoters of interest include the β-actin promoter, SV40 early and late promoters, immunoglobulin promoter, human cytomegalovirus promoter, and the Friend spleen focus-forming virus promoter. The promoters may or may not be associated with enhancers, where the enhancers may be naturally associated with the particular promoter or associated with a different promoter.

The sequence of the open reading frame may be obtained from genomic DNA, cDNA, or be synthesized, or combinations thereof. Depending upon the size of the genomic DNA and the number of introns, one may wish to use cDNA or a combination thereof. In many instances, it is found that introns stabilize the mRNA. Also, one may provide for non-coding regions which stabilize the mRNA.

A termination region will be provided 3' to the cytoplasmic domain, where the termination region may be naturally associated with the cytoplasmic domain or may be derived from a different source. For the most part, the termination regions are not critical and a wide variety of termination regions may be employed without adversely affecting expression.

The various manipulations may be carried out in vitro or may be introduced into vectors for cloning in an appropriate host, e.g., *E. coli.* Thus, after each manipulation, the resulting construct from joining of the DNA sequences may be cloned into an expression vector. The sequence may be screened by restriction analysis, sequencing, or the like to insure that it encodes the desired chimeric protein.

The chimeric construct may be introduced into the target cell in any convenient manner. Techniques include calcium phosphate or DEAE-dextran mediated DNA transfection, electroporation, protoplast fusion, liposome fusion, biolistics using DNA-coated particles, and infection, where the chimeric construct is introduced into an appropriate virus (e.g. retrovirus, adenovirus, adeno-associated virus, Herpes virus, Sindbis virus, papilloma virus), particularly a non-replicative form of the virus, or the like. In addition, direct injection of naked DNA or protein- or lipid-complexed DNA may also be used to introduce DNA into cells.

Once the target host has been transformed, integration will usually result. However, by appropriate choice of vectors, one may provide for episomal maintenance. A large number of vectors are known which are based on viruses, where the copy number of the virus maintained in the cell is low enough to maintain the viability of the cell. Illustrative vectors include SV40, EBV and BPV.

It is also contemplated that the introduction of the chimeric constructs of the present invention into cells may result in the transient expression of the MSCRs. Such transient expression may be preferable if a short-term therapeutic effect is desired. Unstable replication or the absence of DNA replication may result, for example, from adenovirus infection or transformation with naked DNA.

The MSCRs of the present invention are employed in a wide variety of target host cells, normally cells from vertebrates, more particularly, mammals, desirably domestic animals or primates, particularly humans. In particular, the subject invention may also find application in regulating the expansion and effector activity of lymphoid cells, e.g., T lymphocytes (including CD8+ T cells, CD4+ T cells, cytotoxic T cells and helper T cells), B lymphocytes, cytotoxic lymphocytes (CTL), natural killer cells (NK), tumor-infiltrating-lymphocytes (TIL) or other cells which are capable of killing target cells when activated. In addition, suitable host cells in which to introduce MSCRs of the present invention include hematopoietic stem cells, which develop into cytotoxic effector cells with both myeloid and lymphoid phenotype including granulocytes, mast cells, basophils, macrophages, natural killer (NK) cells and T and B lymphocytes.

Once one has established that the transformed host cell expresses the MSCRs of the present invention in accordance with the desired regulation and at a desired level, one may then determine whether the MSCPRs or hybrid MSCRs are functional in the host cell in providing for the desired proliferation signal. One may use established methodology for measuring proliferation to verify the functional capability of the above MSCRs. The proliferative response of cells can be measured by a variety of techniques known to those skilled in the art. For example, DNA synthesis can be measured by the incorporation of either tritiated thymidine or orotic acid. The incorporation of bromodeoxyuridine into newly synthesized DNA can be measured by immunological staining and the detection of dyes, or by ELISA (Enzyme-linked immunosorbent assay)(Doyle et al., *Cell and Tissue Culture: Laboratory Procedures,* Wiley, Chichester, England, (1994)). The mitotic index of cells can be determined by staining and microscopy, by the fraction labeled mitoses method or by FACS analysis (Doyle et al., supra, (1994); Dean, *Cell Tissue Kinet.* 13:299–308 (1980); Dean, *Cell Tissue Kinet.* 13:672–681 (1980)). The increase in cell size which accompanies progress through the cell cycle can be measure by centrifugal elutriation (Faha et al., *J Virol.* 67:2456–2465 (1993)). Increases in the number of cells may also be measured by counting the cells, with or without the addition of vital dyes. In addition, signal transduction can also be measured by the detection of phosphotyrosine, the in vitro activity of tyrosine kinases from activated cells, c-myc induction, and calcium mobilization as described in the Examples below. In the case of MSCRs containing EFSDs, one may determine whether the host cell has been provided with an effector signal in a variety of ways well known to those skilled in the art, depending on the EFSD and the cell type. For example, the activity of MSCEFRs and hybrid MSCRs in signaling cytotoxic effector function in engineered cytotoxic T cells can be measured by the release of ^{51}Cr from labeled cells displaying extracellular inducer molecules, while the activity of MSCEFRs and hybrid MSCRs in signaling helper effector function in engineered helper T cells can be measured by the release of cytokines in the presence of inducer.

In the present invention, a host cell may express two different MSCRs, one containing an effector function signaling domain and the other containing a proliferation signaling domain (i.e. MSCEFR and MSCPR). Both MSCRs may contain the same MSECDs. Alternatively, a host cell may express a hybrid MSCR combining both signaling domains (EFSD and PSD) on the same chain. In both situations, the binding of an extracellular inducer molecule to the MSECD will stimulate host cells to act as therapeutic agents at the same time they are expanding in response to binding to an extracellular inducer molecule, e.g., gp120 for HIV or cancer-specific antigens.

The specific targets of cells expressing the multispecific chimeric receptors (MSCRs) of the present invention include diseased cells, such as cells infected with HIV, HTLV-I or II, cytomegalovirus, hepatitis A, B or C viruses, *Mycobacterium avium, Mycobacterium tuberculosis, Mycobacterium leprae* etc., neoplastic cells, or autoimmune disease-causing cells where the diseased cells have a surface marker associated with the diseased state. Since MSCRs of the present invention have more than one antigen-binding and/or ligand-binding domain, the present invention may have advantages over monospecific chimeric receptors in treating human diseases such as infectious disease, cancer and autoimmune disease. The application of the present invention for targeting more than one epitope of a given pathogen, more than one pathogen, or a heterogeneous population of disease-causing cells that may be found in malignant or autoimmune disease, is designed to have the therapeutic benefit of a combination therapy, while having the safety profile and pharmacological properties of a single therapeutic agent.

The MSCRs of the present invention are envisioned as a strategy for overcoming the ability of viruses such as HIV to become drug-resistant, and may be applicable in treating other diseases in which drug-resistance or antigenic variation is a significant problem (eg. cancer, bacterial and parasitic infections). Through the application of the present invention, the probability that a given viral variant will eventually arise which is not targeted by a chimeric receptor expressing cell would decrease in direct relation to the number of antigenic specificities recognized by the multispecific chimeric receptor. In particular, single-chain antibodies which recognize many different HIV antigens can be employed to create MSCRs to use as anti-AIDS therapeutics. Similar strategies can be applied for other types of pathogens, such as chronic or recurrent bacterial infections, for which the the problem of drug resistance in the face of ongoing antibiotic therapy is particularly acute.

The MSCRs of the present invention may also be useful in the development of chimeric receptor-expressing cells for the treatment of multiple infections. For example, an MSCR-containing T cell which recognizes HIV as well as another opportunistic pathogen such as CMV, herpesviruses, etc. can also be used as an anti-AIDS therapeutic. Since immunocompromised individuals are also susceptible to various bacterial infections, neutrophils may also be armed with multispecific chimeric receptors which recognize the major classes of relevant pathogens.

The MSCRs of the present invention may also be useful in treating viral and bacterial infections where antigenic variation and the existence of multiple strains has limited traditional, single agent therapies. For example, MSCRs containing MSECDs which recognize multiple antigens or epitopes from a single pathogen can be used to treat HIV, hepatitis A, B and C viruses, Kaposi's sarcoma-associated herpes virus, Herpes Simplex viruses, Herpes Zoster virus, CMV, papilloma virus, respiratory syncytial virus, and influenza viruses.

The MSCR-containing immune cells of the present invention may also find application in cancer therapy, since resistance to chemotherapeutic agents has been an important obstacle in the successful use of traditional treatment regimens and combination chemotherapy has been limited by the significant side effects of these agents and the development of multidrug resistance. In addition, the high growth rate of malignant cells increases the potential for the selection of antigenic variants or for resistance due to antigen or epitope loss. As an example, B cell lymphoma can be treated with MSCRs which recognize more than one pan-B cell surface marker such as CD19, CD20 or CD22, and/or markers specific for malignant but not resting B cells, such as the interleukin-14 receptor.

The present invention may also be useful as a cancer therapy in the MSCR's ability to target cytotoxic cells to cancers such as melanoma in which the host can mount a tumor antigen-specific T cell response. The availability of CTL clones from patients to MHC-restricted epitopes on melanoma has permitted the molecular analysis of the T cell receptors responsible for tumor killing (Mandelboim et al., *Nature,* 369:67–71 (1994); Cox et al., *Science,* 264:716–719 (1994)). However, in employing a single-chain T cell receptor (scTCR) of sufficient affinity to redirect cytolysis, a given chimeric receptor would still be MHC-restricted and active in only a small fraction of the patient population. Multispecific scTCR's could be developed which would recognize the relevant tumor-specific peptide antigen in the context of many HLA haplotypes. Such 'semi-universal' receptors may be capable of dealing with a particular disease target in most affected individuals.

The MSCR technology may also be used to treat autoimmune disorders such as multiple sclerosis, rheumatoid arthritis, type 1 diabetes mellitus, myasthenia gravis and Graves' disease, where the autoreactive T cells and/or B cells are oligo- or polyclonal. Immune cells bearing MSCRs which recognize the members of the restricted T cell receptor (TCR) repertoire expressed by the disease-causing autoreactive T cells can be used to treat autoimmune disease. In particular, restricted TCR expression has been observed for the T cells found at the sites of disease in multiple sclerosis (brain) (Oksenberg et al., *Proc. Natl. Acad. Sci,* 86:988–992 (1989)) and rheumatoid arthritis (joint synovium) (Stamenkovic et al., *Proc. Natl. Acad. Sci.,* 85:1179–1183 (1988)).

High-titer retroviral producer lines are used to transduce the chimeric proliferation receptor constructs into autologous or allogeneic human T-cells, hematopoietic stem cells or other cells, described above through the process of retroviral mediated gene transfer as described by Lusky et al. in (1992) *Blood* 80:396. In addition to the gene encoding the chimeric proliferation receptor, additional genes may be included in the retroviral construct. These include genes such as the thymidine kinase or cytosine deaminase genes (Borrelli et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:7572) which acts as a suicide gene for the marked cells if the patient is exposed to gancyclovir or 5'-fluorouracil (5FU), respectively. Thus, if the percentage of marked cells is too high, gancyclovir or 5FU may be administered to reduce the percentage of cells expressing the chimeric receptors. In addition, if the percentage of marked cells needs to be increased, the multi-drug resistance gene can be included (Sorrentino et al. (1992) *Science* 27:99) which functions as a preferential survival gene for the marked cells in the patients if the patient is administered a dose of a chemotherapeutic agent such as taxol. Therefore, the percentage of marked cells in the patients can be titrated to obtain the maximum therapeutic benefit.

In addition, high-titer retroviral, adenoviral or other viral or non-viral producer lines may be used to transduce the chimeric proliferation receptor constructs into autologous or allogeneic nerve cells, hematopoietic cells including stem cells, islets of Langerhans, keratinocytes, muscle cells or other cells following the methods of retroviral, adenoviral or other viral or non-viral mediated gene transfer as described in Finer et al., *Blood* 83:43–48 (1994) and U.S. patent application Ser. No. 08/333,680. Similar to the procedure described above, other genes may be included in the retroviral, adenoviral or other viral or non-viral constructs in addition to the chimeric proliferation receptor in the recipient cell. After introduction of the construct into the cell type of interest, the cells may be expanded in an appropriate medium well known in the art and used in a variety of ways previously described.

The following examples are by way of illustration and not by way of limitation.

EXAMPLES

Example 1

Multispecific antibodies comprising multiple antibody extracellular clustering domains and an Ig-Fc effector function domain.

Multispecific antibodies were created which contain two or more extracellular clustering domains (ECDs) which are derived from antibodies and/or single-chain antibodies, or modifications thereof. The constructs described in this example contain two SCFv domains which were derived from the 98.6 human monoclonal antibody (Mab), which is specific for the HIV-1 gp41 envelope glycoprotein, and the 447D human MAb, which is specific for the HIV-1 gp120 envelope glycoprotein. The 98.6 light-linker-heavy (LLH) SCFv consists of (from N- to C-terminus): 1) the Vκ signal sequence and VK variable domain (residues 1–107 of the mature protein) of the 98.6 MAb, 2) the 14 amino acid L212 peptide linker (Gly-Ser-Thr-Ser-Gly-Ser-Gly-Lys-Ser-Ser-Glu-Gly-Lys-Gly) (SEQ ID NO:23) (Bedzyk et al *J.Biol Chem.* (1990) 265: 18615–18620), and 3) the VH variable domain (residues 1–113 of the mature protein) of the 98.6 MAb. The 447D light-linker-heavy (LLH) SCFv consists of (from N- to C-terminus): 1) the Vλ signal sequence and Vλ variable domain (residues 1–107 of the mature protein) of the 447D MAb, 2) the 14 amino acid L212 peptide linker, and 3) the VH variable (residues 1–113 of the mature protein) of the 447D MAb. Alternatively, the 98.6 and 447D SCFv's are created as heavy-linker-light (HLL) constructs in which the heavy chain variable domain precedes the light chain variable domain, connected by a suitable oligo- or polypeptide linker. Both LLH or HLL SCFv derivatives of the 98.6 and 447D MAbs may be constructed using a variety of oligo- and polypeptide linkers. In this example, the 98.6 LLH SCFv was joined at its C-terminus (residue 113 of the VH variable domain) to the N-terminus of the 447D LLH SCFv (residue 1 of the Vλ variable domain). Alternatively, the 447D LLH SCFv is joined at its C-terminus (residue 113 of the VH variable domain) to the N-terminus of the 98.6 LLH SCFv (residue 1 of the Vκ variable domain). Either LLH SCFv may be substituted for by the corresponding HLL SCFv, or modifications thereof. The two SCFv's were joined either directly, or via an oligo- or polypeptide linker. The C-terminus of the second SCFv was fused in turn to the hinge and Fc region (residues 226–478) of the human IgG2 heavy chain. Mammalian expression vectors for the 98.6 SCFv/447D SCFv multispecific antibodies described in this example were constructed using plasmid pMSAb1int. This

intermediate plasmid was constructed from three DNA fragments: 1) a 3.9 kb vector fragment obtained by digestion of pIK1.1 with EcoRI and SfiI, 2) a 1.0 kb fragment encoding the 98.6 LLH SCFv domain, obtained by digestion of pIK98.6KLHγ2 with PmlI, modification of the cohesive end with T4 DNA polymerase and dNTPs to create a blunt end, followed by digestion with EcoRI, and 3) a 1.9 kb fragment encoding the 447D LLH SCFv domain and the human IgG2 hinge and Fc domains, obtained by digestion of pIK447DLLHγ2 with EcoRI, modification of the cohesive end with T4 DNA polymerase and dNTPs to create a blunt end, followed by digestion with SfiI. pMSAb1int was identified by restriction enzyme analysis and used to prepare single-stranded DNA template for oligonucleotide-directed mutagenesis. In each example, the correct expression plasmid was identified by restriction mapping and its structure was confirmed by DNA sequencing.

Example 1A

SAb(αgp41)-SAb(αgp120)-Fc

pIK-SAb(αgp41)-SAb(αgp120)-Fc directs the expression of a hybrid protein consisting of the Vκ signal sequence and SCFv domain of 98.6-LLH joined at its C-terminus (98.6-VH residue 113) to the N-terminus of the SCFv domain of 447D-LLH (447D-Vλ residue 1), followed by the human IgG2 hinge and Fc domains (residues 226–478). This plasmid is constructed by oligonucleotide-directed mutagenesis using single-stranded pMSAb1int DNA as the template with oligonucleotide 1 (SEQ ID NO:1) as the primer. The correct expression plasmid was identified by colony hybridization using oligonucleotide 2 (SEQ ID NO:2)as a probe.

Example 1B

SAb(αgp41)-L1-SAb(αgp120)-Fc

pIK-SAb(αgp41)-L1-SAb(αgp120)-Fc directs the expression of a hybrid protein consisting of the Vκ signal sequence and SCFv domain of 98.6-LLH joined at its C-terminus (98.6-VH residue 113) by a 14 amino acid linker (Gly-Ser-Thr-Ser-Gly-Ser-Gly-Lys-Ser-Ser-Glu-Gly-Lys-Gly) (SEQ ID NO:23) to the N-terminus of the SCFv domain of 447D-LLH (447D-Vλ residue 1), followed by the human IgG2 hinge and Fc domains (residues 226–478). This plasmid is constructed by oligonucleotide-directed mutagenesis using single-stranded pMSAb1int DNA as the template with oligonucleotide 3 (SEQ ID NO:3)as the primer. The correct expression plasmid was identified by colony hybridization using oligonucleotide 4 (SEQ ID NO:4)as a probe.

Example 1C

SAb(αgp41)-L2-SAb(αgp120)-Fc

pIK-SAb(αgp41)-L2-SAb(αgp120)-Fc directs the expression of a hybrid protein consisting of the Vκ signal sequence and SCFv domain of 98.6-LLH joined at its C-terminus (98.6-VH residue 113) by a 25 amino acid linker (Ser-Ser-Ala-Asp-Asp-Ala-Lys-Lys-Asp-Ala-Ala-Lys-Lys-Asp-Asp-Ala-Lys-Lys-Asp-Asp-Ala-Lys-Lys-Asp-Gly) (SEQ ID NO:24) to the N-terminus of the SCFv domain of 447D-LLH (447D-Vλ residue 1), followed by the human IgG2 hinge and Fc domains (residues 226–478). This plasmid is constructed by oligonucleotide-directed mutagenesis using single-stranded pMSAb1int DNA as the template with oligonucleotide 5 (SEQ ID NO:5)as the primer. The correct expression plasmid was identified by colony hybridization using oligonucleotide 6 (SEQ ID NO:6)as a probe.

Example 1D

SAb(αgp41)-L3-SAb(αgp120)-Fc

pIK-SAb(αgp41)-L3-SAb(αgp120)-Fc directs the expression of a hybrid protein consisting of the Vκ signal sequence and SCFv domain of 98.6-LLH joined at its C-terminus (98.6-VH residue 113) by a 10 amino acid linker (Asp-Lys-Thr-His-Thr-Ser-Pro-Pro-Ser-Pro) (SEQ ID NO:25) to the N-terminus of the SCFv domain of 447D-LLH (447D-Vλ residue 1), followed by the human IgG2 hinge and Fc domains (residues 226–478). This plasmid is constructed by oligonucleotide-directed mutagenesis using single-stranded pMSAb1int DNA as the template with oligonucleotide 7 (SEQ ID NO:7)as the primer. The correct expression plasmid was identified by colony hybridization using oligonucleotide 8 (SEQ ID NO:8) as a probe.

Example 1E

SAb(αgp41)-L4-SAb(αgp120)-Fc

pIK-SAb(αgp41)-L4-SAb(αgp120)-Fc directs the expression of a hybrid protein consisting of the Vκ signal sequence and SCFv domain of 98.6-LLH joined at its C-terminus (98.6-VH residue 113) by an 18 amino acid linker (Glu-Leu-Gln-Leu-Glu-Glu-Ser-Ser-Ala-Glu-Ala-Gln-Asp-Gly-Glu-Leu-Asp) (SEQ ID NO:26) to the N-terminus of the SCFv domain of 447D-LLH (447D-Vλ residue 1), followed by the human IgG2 hinge and Fc domains (residues 226–478). This plasmid is constructed by oligonucleotide-directed mutagenesis using single-stranded pMSAb1int DNA as the template with oligonucleotide 9 (SEQ ID NO:9) as the primer. The correct expression plasmid was identified by colony hybridization using oligonucleotide 10 (SEQ ID NO:10) as a probe.

Example 2

Multispecific antibodies comprising an antibody extracellular clustering domain, a ligand-receptor (CD4) extracellular clustering domain, and an Ig-Fc effector function domain.

Multispecific antibodies were created which contain two or more extracellular clustering domains (ECDs), at least one of which is derived from an antibody and/or single-chain antibody, or modifications thereof, and at least one of which is derived from a ligand-receptor binding domain, or modifications thereof. The constructs described in this example contain the 447D LLH SCFv domain (described in example 1), and the human CD4 V1 & V2 domains, which bind with high affinity to the HIV-1 gp120 envelope glycoprotein. Alternatively, the 447D SCFv's are created as HLL constructs in which the heavy chain variable domain precedes the light chain variable domain, connected by a suitable oligo- or polypeptide linker. Both LLH or HLL SCFv derivatives of the 447D MAb may be constructed using a variety of oligo- and polypeptide linkers. Other portions of CD4 may be employed in such constructs including the entire CD4 EXT domain (residues 1–371 of the mature polypeptide) as well as various truncations and/or modifications thereof. In this example, the 447D LLH SCFv was joined at its C-terminus (residue 113 of the VH variable domain) to the N-terminus of the CD4 protein (residue 1 of the mature polypeptide). Alternatively, the CD4 protein is joined at the C-terminus of its EXT domain (residue 371 of the mature polypeptide, or truncations thereof, e.g. residue 180 which resides at the C-terminus of the CD4 V1 & V2 domains) to the N-terminus of the 447 LLH SCFv (residue 1 of the Vλ variable domain). The 447D LLH SCFv may be substituted for by the corresponding HLL SCFv, or modifications thereof. The 447D LLH SCFv was joined to the CD4 protein either directly, or via an oligo- or polypeptide linker. The C-terminus of the CD4 V1 & V2 domains, was fused in turn to the hinge and Fc region (residues 226–478) of the human IgG2 heavy chain. Mammalian expression vectors for the 447D SCFv/CD4 V1V2 multispecific antibodies described in this example were constructed using

plasmid pMSAb2int. This intermediate plasmid was constructed from three DNA fragments: 1) a 3.9 kb vector fragment obtained by digestion of pIK1.1 with EcoRI and SfiI, 2) a 1.0 kb fragment encoding the 447D LLH SCFv domain, obtained by digestion of pIK447DLLHγ2 with PmlI, modification of the cohesive end with T4 DNA polymerase and dNTPs to create a blunt end, followed by digestion with EcoRI, and 3) a 1.9 kb fragment encoding the CD4 V1 & V2 domains linked at their C-terminus to the hinge and Fc regions of the human IgG2 heavy chain, obtained by digestion of pIKCD4γ2 with EcoRI, modification of the cohesive end with T4 polymerase and dNTPs to create a blunt end, followed by digestion with SfiI. pMSAb2int was identified by restriction analysis, and used to prepare single-stranded DNA template for oligonucleotide-directed mutagenesis. In each example, the correct expression plasmid was identified by restriction mapping and its structure was confirmed by DNA sequencing.

Example 2A

SAb(αgp120)-CD4-Fc

pIK-SAb(αgp120)-CD4-Fc directs the expression of a hybrid protein consisting of the Vλ signal sequence and SCFv domain of 447D-LLH joined at its C-terminus (447D-VH residue 113) to the N-terminus of human CD4 (residues 1–180 of the mature polypeptide), followed by the human IgG2 hinge and Fc domains (residues 226–478). This plasmid is constructed by oligonucleotide-directed mutagenesis using single stranded pMSAb2int DNA as the template with oligonucleotide 11 (SEQ ID NO:12) as the primer. The correct expression plasmid was identified by colony hybridization using oligonucleotide 12 (SEQ ID NO:12)as a probe.

Example 2B

SAb(αgp120)-L1-CD4-Fc

pIK-SAb(αgp120)-L1-CD4-Fc directs the expression of a hybrid protein consisting of the Vλ signal sequence and SCFv domain of 447D-LLH joined at its C-terminus (447D-VH residue 113) by a 14 amino acid linker (Gly-Ser-Thr-Ser-Gly-Ser-Gly-Lys-Ser-Ser-Glu-Gly-Lys-Gly) (SEQ ID NO:23) to the N-terminus of human CD4 (residues 1–180 of the mature polypeptide), followed by the human IgG2 hinge and Fc domains (residues 226–478). This plasmid is constructed by oligonucleotide-directed mutagenesis using single stranded pMSAb2int DNA as the template with oligonucleotide 13 (SEQ ID NO:13)as the primer. The correct expression plasmid was identified by colony hybridization using oligonucleotide 4 (SEQ ID NO:4)as a probe.

Example 2C

SAb(αgp120)-L2-CD4-Fc

pIK-SAb(αgp120)-L2-CD4-Fc directs the expression of a hybrid protein consisting of the Vλ signal sequence and SCFv domain of 447D-LLH joined at its C-terminus (447D-VH residue 113) by a 25 amino acid linker (Ser-Ser-Ala-Asp-Asp-Ala-Lys-Lys-Asp-Ala-Ala-Lys-Lys-Asp-Asp-Ala-Lys-Lys-Asp-Asp-Ala-Lys-Lys-Asp-Gly) (SEQ ID NO:24) to the N-terminus of human CD4 (residues 1–180 of the mature polypeptide), followed by the human IgG2 hinge and Fc domains (residues 226–478). This plasmid is constructed by oligonucleotide-directed mutagenesis using single stranded pMSAb2int DNA as the template with oligonucleotide 14 (SEQ ID NO:14)as the primer. The correct expression plasmid was identified by colony hybridization using oligonucleotide 6 (SEQ ID NO:6)as a probe.

Example 2D

SAb(αgp120)-L3-CD4-Fc

pIK-SAb(αgp120)-L3-CD4-Fc directs the expression of a hybrid protein consisting of the Vλ signal sequence and SCFv domain of 447D-LLH joined at its C-terminus (447D-VH residue 113) by a 10 amino acid linker (Asp-Lys-Thr-His-Thr-Ser-Pro-Pro-Ser-Pro) (SEQ ID NO:25) to the N-terminus of human CD4 (residues 1–180 of the mature polypeptide), followed by the human IgG2 hinge and Fc domains (residues 226–478). This plasmid is constructed by oligonucleotide-directed mutagenesis using single stranded pMSAb2int DNA as the template with oligonucleotide 15 (SEQ ID NO:15) as the primer. The correct expression plasmid was identified by colony hybridization using oligonucleotide 8 (SEQ ID NO:8) as a probe.

Example 2E

SAb(αgp120)-L4-CD4-Fc

pIK-SAb(αgp120)-L4-CD4-Fc directs the expression of a hybrid protein consisting of the Vλ signal sequence and SCFv domain of 447D-LLH joined at its C-terminus (447D-VH residue 113) by an 18 amino acid linker (Glu-Leu-Gln-Leu-Glu-Glu-Ser-Ser-Ala-Glu-Ala-Gln-Asp-Gly-Glu-Leu-Asp) (SEQ ID NO:26) to the N-terminus of human CD4 (residues 1–180 of the mature polypeptide), followed by the human IgG2 hinge and Fc domains (residues 226–478). This plasmid is constructed by oligonucleotide-directed mutagenesis using single stranded pMSAb2int DNA as the template with oligonucleotide 16 (SEQ ID NO:16) as the primer. The correct expression plasmid was identified by colony hybridization using oligonucleotide 10 (SEQ ID NO:10) as a probe.

Example 3

Expression & characterization of multispecific antibodies

To determine whether each multispecific antibody can be efficiently expressed and secreted, and thus properly folded, each corresponding mammalian expression vector was transfected into a model mammalian cell, using the human 293 embryonic kidney cell line (ATCC CRL 1573). Following transfection, the expression and corresponding apparent molecular mass of each polypeptide was evaluated by radioimmunoprecipitation (RIP), and the level of secretion was quantitated using an enzyme-linked immunosorbent assay (ELISA).

Example 3A

Transfection of human 293 cells with multispecific antibody expression vectors.

For transfection, 293 cells were grown in DMEM:F12 media (JRH Biosciences) containing 10% fetal calf serum, and passaged at a 1:8 to 1:12 split ratio every 3 to 4 days. Forty-eight hours prior to transfection, cells were plated by passaging the contents of one subconfluent 10 cm tissue culture dish onto twenty 6 cm tissue culture dishes. Five μg of each expression plasmid DNA was transfected onto a 6 cm dish by the calcium phosphate coprecipitation method (Wigler et al. (1979) *Cell* 16:777). Sixteen hours after transfection, the transfected cells were fed with fresh complete medium. The expression of multispecific antibody polypeptides was evaluated by RIP analysis and ELISA at 48 hours post-transfection.

Example 3B

Radioimmunoprecipitation analysis of multispecific antibodies expressed in transfected 293 cells

Forty hours after transfection, 293 cells were fed with 2 ml of methionine- and cysteine-deficient RPMI media con-

taining 10% dialysed fetal calf serum, supplemented with [35S]-methionine and [35S]-cysteine (Tran35Slabel, 1160 Ci/mMol, ICN Biomedicals, Inc., Irvine, Calif.). Cells were cultured for an additional 8 hours, the conditioned medium harvested, and the labelled cells lysed in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)). For radio-immunoprecipitation, labelled conditioned medium was mixed with an equal volume of 2X RIPA buffer. 35S-labelled multispecific antibodies, which bind to the Staphylococcus aureus Protein A via their IgG2 Fc domain, were then precipitated with 10 μl Pansorbin (Calbiochem, La Jolla, Calif.). Immunoprecipitates were washed three times in RIPA buffer and once in distilled water, and boiled for several minutes in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 150 mM β-mercaptoethanol, 2% SDS, 10% glycerol). Immunoprecipitates were also analysis following boiling in non-reducing SDS sample buffer (lacking β-mercaptoethanol). Samples were analysed by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE). Gels were fixed in 20% methanol and 10% acetic acid, soaked in Enlightening solution (NEN Research Products, Boston, MA) for 15 minutes, dried and subjected to autoradiography. This analysis revealed that multispecific antibodies of the predicted molecular mass are expressed and secreted into the culture medium (FIG. 3). Samples analysed by SDS-PAGE under non-denaturing conditions revealed multispecific antibodies with the molecular mass expected for the corresponding homodimeric proteins.

Example 3C

ELISA analysis of multispecific antibodies secreted by transfected 293 cells

Human 293 cells were transfected as described above and were fed fresh medium 16 hours later. Forty-eight hours after transfection the conditioned medium from the transfected cells was harvested and analysed by ELISA. Nunc Maxisorp microtiter plates (Nunc Inc., Naperville, Ill.) were coated for 16 hours at 4° C. with an (Fab')2 fragment derived from a rabbit anti-human IgG polyclonal antisera (Accurate Chemical and Scientific Corporation Westbury, N.Y.), that was diluted 1:1000 in 0.05 M sodium carbonate buffer pH 9.6. Plates were then washed three times in PBS containing 0.05% Tween-20 (PBS-Tween), followed by blocking with PBS containing 1% bovine serum albumin (PBSA) at room temperature for 1 hour. Human IgG2 (Calbiochem, La Jolla, Calif.) was employed as a positive control standard in the assays. Samples and standards were diluted in PBSA, added to the antibody-coated plates, and the plates then incubated for 16 hours at 4° C. Plates were washed three times with PBS-Tween, and an enzyme-linked detection antibody was added to the plates and incubated for 1 hour at room temperature. The detection antibody used was horseradish peroxidase-conjugated goat anti-human IgG polyclonal antisera (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) that was diluted 1:50,000 in PBSA. Plates were then washed 3 times with PBS-Tween and an indicator solution consisting of 1.7 mg/ml o-phenylenediamine hydrochloride in 16 mM citrate buffer pH 4.5 was added. Color development was stopped by the addition of 1 M sulfuric acid, and the results read on a UVmax microplate reader (Molecular Devices Corporation, Menlo Park, Calif.). As shown in Table 1, significant levels of each of the multispecific antibodies described in Examples 1A–E and 2A–E were detected in the culture supernatants of transfected 293 cells.

TABLE 1

Construct	Concentration (μg/ml)
CD4-Fc	1.6
SAb(αgp41)-Fc	1.2
SAb(αgp120-Fc	1.3
SAb(αgp41)-SAb(αgp120)-Fc	0.23
SAb(αgp41)-L1-SAb(αgp120)-Fc	0.65
SAb(αgp41)-L2-SAb(αgp120)-Fc	0.75
SAb(αgp41)-L3-SAb(αgp120)-Fc	0.40
SAb(αgp41)-L4-SAb(αgp120)-Fc	0.48
SAb(αgp120)-CD4-Fc	0.05
SAb(αgp120)-L1-CD4-Fc	0.17
SAb(αgp120)-L2-CD4-Fc	0.19
SAb(αgp120)-L3-CD4-Fc	0.10
SAb(αgp120)-L4-CD4-Fc	0.12

Example 4

Ligand/antigen binding properties of multispecific antibodies

Multispecific antibodies based upon the HIV-1 binding domains of CD4 and the 447D and 98.6 MAbs are further characterized for their ability to bind to the HIV-1 gp120 and gp41 proteins which are the proteolytic cleavage products of the gp160env precursor protein, using cell lines which express wild-type gp160env or mutant forms of gp160env in which one or another binding epitope is impaired.

To facilitate this analysis, cell lines are generated which efficiently express surface gp120 and gp41 polypeptides. Mutants of gp160env are generated which have significantly diminished binding to at least one of the ECDs present in a given multispecific antibody to permit detection of binding mediated by the other ECD(s) present. Levels of binding are determined by FACS analysis using the multispecific antibodies to stain the gp160env-expressing cells.

Example 4A

Vectors for efficient expression of HIV-1 gp160env

pIKenv+/rev+/tat− is a vector designed to allow the efficient expression of gp160env in mammalian cells, based on the observation that while expression of the rev gene product is essential for efficient expression of gp160env, expression of the tat gene product may be inhibitory (Bird et al. *J. Biol. Chem.* (1990) 265: 19151–19157). This plasmid was made in two steps. The first step was the construction of pIKenv, which directs the expression of three HIV gene products: env, rev and tat. This plasmid was constructed from two DNA fragments: 1) a 4.3 kb vector fragment obtained by digestion of pIK1.1 with BglII, modification of the cohesive end with T4 DNA polymerase and dNTPs to create a blunt end, followed by digestion with EcoRI, and 2) a 3.1 kb fragment encoding the env, rev and tat gene products of the HXB2 isolate of HIV-1, obtained by digestion of pCMVenv (U.S. Pat. No. 5,359,046) with XhoI, modification of the cohesive end with T4 DNA polymerase and dNTPs to create a blunt end, followed by digestion with EcoRI. pIKenv was identified by restriction analysis, and used in the second step to prepare a single-stranded DNA template for oligonucleotide-directed mutagenesis using oligonucleotide 17 (SEQ ID NO:17)as a primer to remove the initiator methionine codon (ATG) and the adjacent arginine codon (GAG) of the tat gene, and to replace these sequences with a novel PstI site (CTGCAG). The correct plasmid, pIKenv+/rev+/tat−, was identified by colony hybridization using oligonucleotide 18 (SEQ ID NO:18)as a probe and its structure was confirmed by DNA sequencing.

Example 4B

Expression of an HIV-1 gp160env gene with a mutation in the CD4 binding site

pIKenvG370R/rev+/tat- directs the expression of a mutant gp160env polypeptide in which Glu-370 is replaced with Arg, which results in a loss of CD4 binding (Olshevsky et al., *J. Virol.* 64: 5701- 5707). This plasmid is constructed by oligonucleotide-directed mutagenesis using single-stranded pIKenv+/rev+/tat- DNA with oligonucleotide 19 (SEQ ID NO:19) as the primer. The correct expression plasmid is identified by colony hybridization using oligonucleotide 20 (SEQ ID NO:20)as a probe, and its structure is confirmed by DNA sequencing.

Example 4C

Expression of an HIV-1 gp160env gene with a mutation in the 447D MAb binding site

pIKenvR315Q/rev+/tat- directs the expression of a mutant gp160env polypeptide in which Arg-315 is replaced with Gln, which results in a loss of 447D MAb binding (Gorny et al, *J. Virol.* 66: 7538–7542). This plasmid is constructed by oligonucleotide-directed mutagenesis using single stranded pIKenv+/rev+/tat- DNA with oligonucleotide 21 (SEQ ID NO:21) as the primer. The correct expression plasmid is identified by colony hybridization using oligonucleotide 22 (SEQ ID NO:22) as a probe, and its structure is confirmed by DNA sequencing.

Example 4D

Binding of multispecific antibodies to mutant and wild-type HIV-1 gp160env polypeptides

Human 293 cells are transfected with vectors directing the expression of the multispecific antibodies described in Examples 1 & 2, as well as vectors directing the expression of the parent monospecific antibodies (pIKCD4γ2 for CD4-Fc, pIK447D-LLHγ2 for 447D-LLH-Fc, and pIK98.6KLHγ2 for 98.6-LLH-Fc). Supernatants from transfected 293 cells are analysed by ELISA to determine the concentration of the recombinant proteins secreted into the culture medium. Known amounts of each recombinant protein are then used to determine their ability to bind to cell surface-expressed wild-type and mutant gp160env polypeptides, by FACS analysis of human 293 cells which have been transfected with pIKenv+/rev+/tat-, pIKenvG370R/rev+/tat- and pIKenvR315Q/rev+/tat-. Following the binding of each multispecific or monospecific antibody, the cells are stained with FITC-conjugated mouse anti-human IgG MAb (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). The difference in the degree of fluorescence intensity obtained with multispecific antibodies versus each of the parent monospecific antibodies on transfected cells expressing either mutant or wild-type gp160env polypeptides reveals the ability of each ECD contained within a given multispecific antibody to bind to gp160env.

Example 5

MSCRs comprising multiple antibody extracellular clustering domains and a ζ family signaling domain.

Multispecific chimeric receptors (MSCRs) were created from multispecific antibodies which contain two or more extracellular clustering domains (ECDs) derived from antibodies and/or single-chain antibodies, or modifications thereof. The constructs described in this example contain two LLH SCFv domains which were derived from the 98.6 and 447D human MAbs, respectively, as described in Example 1. Alternatively, the 98.6 and 447D SCFv's are created as heavy-linker-light (HLL) constructs in which the heavy chain variable domain precedes the light chain variable domain, connected by a suitable oligo- or polypeptide linker. Both LLH or HLL SCFv derivatives of the 98.6 and 447D MAbs may be constructed using a variety of oligo- and polypeptide linkers. In this example, the 98.6 LLH SCFv was joined at its C-terminus (residue 113 of the VH variable domain) to the N-terminus of the 447D LLH SCFv (residue 1 of the Vλ variable domain). Alternatively, the 447D LLH SCFv is joined at its C-terminus (residue 113 of the VH variable domain) to the N-terminus of the 98.6 LLH SCFv (residue 1 of the Vκ variable domain). Either LLH SCFv may be substituted for by the corresponding HLL SCFv, or modifications thereof. The two SCFv's were joined either directly, or via an oligo- or polypeptide linker. The C-terminus of the second SCFv was fused in turn to the hinge and Fc region (residues 226–477) of the human IgG2 heavy chain, followed by (from N- to C-terminus): the 18 residue human IgG3 M1 membrane hinge, the CD4 TM domain (residues 372–395 of the mature polypeptide), and the ζ CYT domain (residues 31–142 of the mature polypeptide). Mammalian transduction-expression vectors for the 98.6 SCFv/447D SCFv MSCRs described in this example were constructed using plasmid pRT43.2F16, a retroviral vector which directs the expression of the SAb-ζ monospecific chimeric receptor F16 (U.S. Pat. No. 5,359,046) which contains the 98.6 LLH SCFv domain fused to (from N- to C-terminus): the Fc domain of human IgG1 heavy chain, the IgG3 M1 membrane hinge domain, the CD4 TM domain, and the ζ CYT domain. Plasmid pRT43.2F16 was constructed from three fragments: 1) a 6.7 kb vector fragment obtained by digestion of pRT43.2F3 (as described in U.S. patent application Ser. No. 08/258,152) with EcoRI and Apa I, 2) a 1.4 kb fragment encoding the 98.6 LLH SCFv domain and the N-terminal portion of the IgG1 Fc domain, obtained by digestion of pIKF16 (U.S. Pat. No. 5,359,046) with EcoRI and NsiI, and 3) a 0.7 kb fragment encoding the remainder of the IgG1 Fc domain, the IgG3 membrane hinge domain, the CD4 TM domain and the ζ CYT domain, obtained by digestion of pIKF16 with NsiI and ApaI. The construction of MSCRs with an IgG2 Fc domain using a monospecific chimeric receptor with an IgG1 Fc domain was facilitated by the fact that the CH3 regions of the human IgG1 and IgG2 Fc domains share a unique NsiI site, and the amino acid sequences of each which follow this restriction site are identical. In each example, the correct expression plasmid was identified by restriction mapping.

Example 5A

SAb(αgp41)-SAb(αgp120)-Fc-ζ

pRT-SAb(αgp41)-SAb(αgp120)-Fc-ζ directs the expression of a hybrid protein consisting of the Vκ signal sequence and SCFv domain of 98.6-LLH joined at its C-terminus (98.6-VH residue 113) to the N-terminus of the SCFv domain of 447D-LLH (447D-Vλ residue 1), followed by (from N- to C-terminus): the human IgG2 hinge and Fc domains (residues 226–478), the IgG3 Ml membrane hinge domain, the CD4 TM domain (residues 372–395 of the mature polypeptide) and the ζ CYT domain (residues 31–142 of the mature polypeptide). This plasmid is constructed from two fragments: 1) a 7.5 kb vector fragment encoding the C-terminal portion of the IgG1 Fc domain (identical to the corresponding IgG2 region), the IgG3 M1 domain, the CD4 TM domain and the ζ CYT domain, obtained by digestion of pIK43.2F16 with EcoRI and NsiI, and 2) a 2.0 kb fragment encoding the 98.6-LLH SCFv domain, the 447D-LLH SCFv domain and the N-terminal portion of the IgG2 Fc domain, obtained by digestion of pIK-SAb(αgp41)-SAb(αgp120)-Fc with EcoRI and NsiI.

Example 5B

SAb(αgp41)-L1-SAb(αgp120)-Fc-ζ

pRT-SAb(αgp41)-L1-SAb(αgp120)-Fc-ζ directs the expression of a hybrid protein consisting of the Vκ signal sequence and SCFv domain of 98.6-LLH joined at its C-terminus (98.6-VH residue 113) by a 14 amino acid peptide linker (Gly-Ser-Thr-Ser-Gly-Ser-Gly-Lys-Ser-Ser-Glu-Gly-Lys-Gly) (SEQ ID NO:23) to the N-terminus of the SCFv domain of 447D-LLH (447D-Vλ residue 1), followed by (from N- to C-terminus): the human IgG2 hinge and Fc domains (residues 226–478), the IgG3 M1 membrane hinge domain, the CD4 TM domain (residues 372–395 of the mature polypeptide) and the ζ CYT domain (residues 31–142 of the mature polypeptide). This plasmid was constructed from two fragments: 1) a 7.5 kb vector fragment encoding the C-terminal portion of the IgG1 Fc domain (identical to the corresponding IgG2 region), the IgG3 M1 domain, the CD4 TM domain and the ζ CYT domain, obtained by digestion of pIK43.2F16 with EcoRI and NsiI, and 2) a 2.0 kb fragment encoding the 98.6-LLH SCFv domain, the 14 amino acid peptide linker, the 447D-LLH SCFv domain and the N-terminal portion of the IgG2 Fc domain, obtained by digestion of pIK-SAb(αgp41)-L1-SAb (αgp120)-Fc with EcoRI and NsiI.

Example 5C

SAb(αgp41)-L2-SAb(αgp120)-Fc-ζ

pRT-SAb(αgp41)-L2-SAb(αgp120)-Fc-ζ directs the expression of a hybrid protein consisting of the Vκ signal sequence and SCFv domain of 98.6-LLH joined at its C-terminus (98.6-VH residue 113) by a 25 amino acid linker (Ser-Ser-Ala-Asp-Asp-Ala-Lys-Lys-Asp-Ala-Ala-Lys-Lys-Asp-Asp-Ala-Lys-Lys-Asp-Asp-Ala-Ala-Lys-Lys-Asp-Gly) (SEQ ID NO:24) to the N-terminus of the SCFv domain of 447D-LLH (447D-Vλ residue 1), followed by (from N- to C-terminus): the human IgG2 hinge and Fc domains (residues 226–478), the IgG3 M1 membrane hinge domain, the CD4 TM domain (residues 372–395 of the mature polypeptide) and the λ CYT domain (residues 31–142 of the mature polypeptide). This plasmid was constructed from two fragments: 1) a 7.5 kb vector fragment encoding the C-terminal portion of the IgG1 Fc domain (identical to the corresponding IgG2 region), the IgG3 M1 domain, the CD4 TM domain and the ζ CYT domain, obtained by digestion of pIK43.2F16 with EcoRI and NsiI, and 2) a 2.1 kb fragment encoding the 98.6-LLH SCFv domain, the 25 amino acid peptide linker, the 447D-LLH SCFv domain and the N-terminal portion of the IgG2 Fc domain, obtained by digestion of pIK-SAb(αgp41)-L2-SAb(αgp120)-Fc with EcoRI and NsiI.

Example 5D

SAb(αgp41)-L3-SAb(αgp120)-Fc-ζ

pRT-SAb(αgp41)-L3-SAb(αgp120)-Fc-ζ directs the expression of a hybrid protein consisting of the Vκ signal sequence and SCFv domain of 98.6-LLH joined at its C-terminus (98.6-VH residue 113) by a 10 amino acid linker (Asp-Lys-Thr-His-Thr-Ser-Pro-Pro-Ser-Pro) (SEQ ID NO:25) to the N-terminus of the SCFv domain of 447D-LLH (447D-Vλ residue 1), followed by (from N- to C-terminus): the human IgG2 hinge and Fc domains (residues 226–478), the IgG3 M1 membrane hinge domain, the CD4 TM domain (residues 372–395 of the mature polypeptide) and the ζ CYT domain (residues 31–142 of the mature polypeptide). This plasmid is constructed from two fragments: 1) a 7.5 kb vector fragment encoding the C-terminal portion of the IgG1 Fc domain (identical to the corresponding IgG2 region), the IgG3 Ml domain, the CD4 TM domain and the ζ CYT domain, obtained by digestion of pIK43.2F16 with EcoRI and NsiI, and 2) a 2.0 kb fragment encoding the 98.6-LLH SCFv domain, the 10 amino acid peptide linker, the 447D-LLH SCFv domain and the N-terminal portion of the IgG2 Fc domain, obtained by digestion of pIK-SAb(αgp41)-L3-SAb(αgp120)-Fc with EcoRI and NsiI.

Example 5E

SAb(αgp41)-L4-SAb(αgp120)-Fc-ζ

pRT-SAb(αgp41)-L4-SAb(αgp120)-Fc-ζ directs the expression of a hybrid protein consisting of the Vκ signal sequence and SCFv domain of 98.6-LLH joined at its C-terminus (98.6-VH residue 113) by an 18 amino acid linker (Glu-Leu-Gln-Leu-Glu-Glu-Ser-Ser-Ala-Glu-Ala-Gln-Asp-Gly-Glu-Leu-Asp) (SEQ ID NO:26) to the N-terminus of the SCFv domain of 447D-LLH (447D-Vλ residue 1), followed by (from N- to C-terminus): the human IgG2 hinge and Fc domains (residues 226–478), the IgG3 M1 membrane hinge domain, the CD4 TM domain (residues 372–395 of the mature polypeptide) and the ζ CYT domain (residues 31–142 of the mature polypeptide). This plasmid is constructed from two fragments: 1) a 7.5 kb vector fragment encoding the C-terminal portion of the IgG1 Fc domain (identical to the corresponding IgG2 region), the IgG3 M1 domain, the CD4 TM domain and the ζ CYT domain, obtained by digestion of pIK43.2F16 with EcoRI and NsiI, and 2) a 2.0 kb fragment encoding the 98.6-LLH SCFv domain, the 18 amino acid peptide linker, the 447D-LLH SCFv domain and the N-terminal portion of the IgG2 Fc domain, obtained by digestion of pIK-SAb(αgp41)-L4-SAb (αgp120)-Fc with EcoRI and NsiI.

Example 6

MSCRs comprising an antibody extracellular clustering domain, a ligand-receptor (CD4) extracellular clustering domain, and a ζ family signaling domain.

Multispecific chimeric receptors (MSCRs) were created from multispecific antibodies which contain two or more extracellular clustering domains (ECDs), at least one of which is derived from an antibody and/or single-chain antibody, or modifications thereof, and at least one of which is derived from a ligand-receptor binding domain, or modifications thereof. The constructs described in this example contain the 447D LLH SCFv domain, and the human CD4 V1–V4 domains, which bind with high affinity to the HIV-1 gp120 envelope glycoprotein. Alternatively, the 447D SCFv's are created as HLL constructs in which the heavy chain variable domain precedes the light chain variable domain, connected by a suitable oligo- or polypeptide linker. Both LLH or HLL SCFv derivatives of the 447D MAb may be constructed using a variety of oligo- and polypeptide linkers. Portions of CD4 other than the entire CD4 EXT domain (residues 1–371 of the mature polypeptide) made be employed, including various truncations and/or modifications thereof. In this example, the 447D LLH SCFv was joined at its C-terminus (residue 113 of the VH variable domain) to the N-terminus of the CD4 protein (residue 1 of the mature polypeptide). Alternatively, the CD4 protein is joined at the C-terminus of its entire EXT domain (residue 371 of the mature polypeptide, or truncations thereof, e.g. residue 180 which resides at the C-terminus of the CD4 V1 & V2 domains) to the N-terminus of the 447 LLH SCFv (residue 1 of the Vλ variable domain). The 447D LLH SCFv may be substituted for by the corresponding HLL SCFv, or modifications thereof. The 447D LLH SCFv was joined to the CD4 protein either directly, or via an oligo- or polypeptide linker. The C-terminus of the CD4 EXT domain, was

fused in turn to the CD4 TM domain (residues 372–395 of the mature polypeptide), and the ζ CYT domain (residues 31–142 of the mature polypeptide). Mammalian transduction-expression vectors for the 447D SCFv/CD4 MSCRs described in this example were constructed using pRT43.2F3, a retroviral vector which directs the expression of the CD4-ζ monospecific chimeric receptor F3 (U.S. Pat. No. 5,359,046) comprised of the CD4 EXT and TM domains fused to the ζ CYT domain. The construction of MSCRs with a C-terminal CD4 EXT domain was facilitated by using a unique NheI restriction site in CD4 which is present in both the 447D SCFv/CD4 multispecific antibody and the CD4ζ monospecific chimeric receptor. In each example, the correct expression plasmid was identified by restriction mapping.

Example 6A

SAb(αgp120)-CD4-ζ

pRT-SAb(αgp120)-CD4ζ directs the expression of a hybrid protein consisting of the Vλ secretion leader and SCFv domain of 447D-LLH joined at its C-terminus (447D-VH residue 113) to the CD4 EXT and TM domains (residues 1–395 of the mature polypeptide) and the ζ CYT domain (residues 31–142 of the mature polypeptide). This plasmid is constructed from three fragments: 1) a 6.7 kb vector fragment obtained by digesting pRT43.2F3 with EcoRI and ApaI, 2) a 1.4 kb fragment encoding the 447D-LLH SCFv domain and the N-terminal portion of the CD4 EXT domain, obtained by digestion of pIKSAb(αgp120)-CD4-Fc with EcoRI and NheI, and 3) a 1.2 kb fragment encoding the C-terminal portion of the CD4 EXT domain, the CD4 TM domain and the ζ CYT domain, obtained by digestion of pIK43.2F3 with NheI and ApaI.

Example 6B

SAb(αgp120)-L1-CD4-ζ

pRT-SAb(αgp120)-L1-CD4ζ directs the expression of a hybrid protein consisting of the Vλ secretion leader and SCFv domain of 447D-LLH joined at its C-terminus (447D-VH residue 113) by a 14 amino acid linker (Gly-Ser-Thr-Ser-Gly-Ser-Gly-Lys-Ser-Ser-Glu-Gly-Lys-Gly) (SEQ ID NO:23) to the CD4 EXT and TM domains (residues 1–395 of the mature polypeptide) and the ζ CYT domain (residues 31–142 of the mature polypeptide). This plasmid was constructed from three fragments: 1) a 6.7 kb vector fragment obtained by digesting pRT43.2F3 with EcoRI and ApaI, 2) a 1.5 kb fragment encoding the 447D-LLH SCFv domain, the 14 amino acid peptide linker and the N-terminal portion of the CD4 EXT domain, obtained by digestion of pIKSAb (αgp120)-L1-CD4-Fc with EcoRI and NheI, and 3) a 1.2 kb fragment encoding the C-terminal portion of the CD4 EXT domain, the CD4 TM domain and the ζ CYT domain, obtained by digestion of pIK43.2F3 with NheI and ApaI.

Example 6C

SAb(αgp120)-L2-CD4-ζ

pRT-SAb(αgp120)-L2-CD4ζ directs the expression of a hybrid protein consisting of the Vλ secretion leader and SCFv domain of 447D-LLH joined at its C-terminus (447D-VH residue 113) by a 25 amino acid linker (Ser-Ser-Ala-Asp-Asp-Ala-Lys-Lys-Asp-Ala-Ala-Lys-Lys-Asp-Asp-Ala-Lys-Lys-Asp-Asp-Ala-Lys-Lys-Asp-Gly) (SEQ ID NO:24) to the CD4 EXT and TM domains (residues 1–395 of the mature polypeptide) and the ζ CYT domain (residues 31–142 of the mature polypeptide). This plasmid was constructed from three fragments: 1) a 6.7 kb vector fragment obtained by digesting pRT43.2F3 with EcoRI and ApaI, 2) a 1.5 kb fragment encoding the 447D-LLH SCFv domain, the 25 amino acid peptide linker and the N-terminal portion of the CD4 EXT domain, obtained by digestion of pIKSAb (αgp120)-L2-CD4-Fc with EcoRI and NheI, and 3) a 1.2 kb fragment encoding the C-terminal portion of the CD4 EXT domain, the CD4 TM domain and the ζ CYT domain, obtained by digestion of pIK43.2F3 with NheI and ApaI.

Example 6D

SAb(αgp120)-L3-CD4-ζ

pRT-SAb(αgp120)-L3-CD4-ζ directs the expression of a hybrid protein consisting of the Vλ secretion leader and SCFv domain of 447D-LLH joined at its C-terminus (447D-VH residue 113) by a 10 amino acid linker (Asp-Lys-Thr-His-Thr-Ser-Pro-Pro-Ser-Pro) (SEQ ID NO:25) to the CD4 EXT and TM domains (residues 1–395 of the mature polypeptide) and the ζ CYT domain (residues 31–142 of the mature polypeptide). This plasmid is constructed from three fragments: 1) a 6.7 kb vector fragment obtained by digesting pRT43.2F3 with EcoRI and ApaI, 2) a 1.4 kb fragment encoding the 447D-LLH SCFv domain, the 10 amino acid peptide linker and the N-terminal portion of the CD4 EXT domain, obtained by digestion of pIKSAb(αgp120)-L3-CD4-Fc with EcoRI and NheI, and 3) a 1.2 kb fragment encoding the C-terminal portion of the CD4 EXT domain, the CD4 TM domain and the ζ CYT domain, obtained by digestion of pIK43.2F3 with NheI and ApaI.

Example 6E

SAb(αgp120)-L4-CD4-ζ

pRT-SAb(αgp120)-L4-CD4-ζ directs the expression of a hybrid protein consisting of the Vλ secretion leader and SCFv domain of 447D-LLH joined at its C-terminus (447D-VH residue 113) by an 18 amino acid linker (Glu-Leu-Gln-Leu-Glu-Glu-Ser-Ser-Ala-Glu-Ala-Gln-Asp-Gly-Glu-Leu-Asp) (SEQ ID NO:26) to the CD4 EXT and TM domains (residues 1–395 of the mature polypeptide) and the ζ CYT domain (residues 31–142 of the mature polypeptide). This plasmid is constructed from three fragments: 1) a 6.7 kb vector fragment obtained by digesting pRT43.2F3 with EcoRI and ApaI, 2) a 1.5 kb fragment encoding the 447D-LLH SCFv domain, the 18 amino acid peptide linker and the N-terminal portion of the CD4 EXT domain, obtained by digestion of pIKSAb(αgp120)-L4-CD4-Fc with EcoRI and NheI, and 3) a 1.2 kb fragment encoding the C-terminal portion of the CD4 EXT domain, the CD4 TM domain and the ζ CYT domain, obtained by digestion of pIK43.2F3 with NheI and ApaI.

Example 7

Multispecific antibodies & MSCRs comprising two antibody extracellular clustering domains, a ligand-receptor (CD4) extracellular clustering domain, and a ζ family signaling domain.

Multispecific antibodies and MSCRs are created which contain three or more extracellular clustering domains (ECDs), at least one of which is derived from an antibody and/or single-chain antibody, or modifications thereof, and at least one of which is derived from a ligand-receptor binding domain, or modifications thereof. The constructs described in this example contain the 98.6 LLH SCFv domain, the 447D LLH SCFv domain, and the human CD4 V1–V2 domains, in the case of the multispecific antibodies, and the entire CD4 EXT domains, in the case of the MSCRs. Alternatively, the 98.6 and 447D SCFv's are created as HLL constructs in which the heavy chain variable domain precedes the light chain variable domain, connected by a suitable oligo- or polypeptide linker. Both LLH or HLL SCFv derivatives of the 98.6 and 447D MAb may be

constructed using a variety of oligo- and polypeptide linkers and substituted accordingly in the multispecific antibodies and MSCRs herein described. Portions of CD4 other than the entire CD4 EXT or V1–V2 domains may similarly be employed, including various truncations and/or modifications thereof. In this example, the order of ECDs is (from N- to C-terminal): the 98.6 LLH SCFv domain, the 447D LLH SCFv domain, and the CD4 EXT domain. Alternatively, it is possible to create variants of all of the possible permutations of the order of these three as well as other domains. The 98.6 LLH SCFv, 447D LLH SCFv, CD4 and other ECDs may be linked either directly, or via various oligo- or polypeptide linkers. In the event that a SCFv is the most C-terminal ECD in an MSCR, it may be fused in turn to the hinge and Fc region (residues 226–477) of the human IgG2 heavy chain, followed by (from N- to C-terminus): the 18 residue human IgG3 M1 membrane hinge, the CD4 TM domain (residues 372–395 of the mature polypeptide), and the ζ CYT domain (residues 31–142 of the mature polypeptide). In the event that CD4 is the most C-terminus ECD of the MSCR, it may be fused in turn to the CD4 TM domain (residues 372–395 of the mature polypeptide), and the ζ CYT domain (residues 31–142 of the mature polypeptide). In each example, the correct expression plasmid was identified by restriction mapping.

Example 7A

SAb(αgp41)-(Lx)-SAb(αgp120)-(Ly)-CD4-Fc

A series of plasmids of the general structure pIKSAb (αgp41)-(Lx)-SAb(αgp120)-(Ly)-CD4-Fc, where Lx and Ly are any one of a number of various oligo- and polypeptide linkers including L1, L2, L3, L4 or no linker, direct the expression of a series of hybrid proteins consisting (from N- to C-terminus) of 1) the Vκ signal sequence and 98.6-LLH SCFv domain, 2) linker Lx, 3) the 447D-LLH SCFv, 4) linker Ly, 5) a portion of the CD4 EXT domain (residues 1–180 of the mature polypeptide), and 5) the human IgG2 hinge and Fc domains (residues 226–478). These plasmids are constructed from three fragments: 1) a 4.3 kb vector fragment obtained by digestion of pIK1.1 with EcoRI and BglII, 2) a 1.7 kb fragment encoding the C-terminus of the 447D-LLH SCFv domain, linker Ly, the CD4 V1 & V2 domains, and the IgG2 Fc domain, obtained by digestion of one of the pIKSAb(αgp120)-Ly-CD4-Fc series of plasmids with SpeI and BglII, and 3) a 1.2 kb fragment encoding the entire 98.6-LLH SCFv domain, linker Lx, and the N-terminus of the 447D-LLH SCFv domain, obtained by digestion of one of the pIKSAb(αgp41)-Lx-SAb(αgp120)-Fc series of plasmids with EcoRI and SpeI.

Example 7B

SAb(αgp41)-(Lx)-SAb(αgp120)-(Ly)-CD4-ζ

A series of plasmids of the general structure pIKSAb (αgp41)-(Lx)-SAb(αgp120)-(Ly)-CD4ζ, where Lx and Ly are any one of a number of various oligo- and polypeptide linkers including L1, L2, L3, L4 or no linker, direct the expression of a series of hybrid proteins consisting (from N- to C-terminus) of 1) the Vκ signal sequence and 98.6-LLH SCFv domain, 2) linker Lx, 3) the 447D-LLH SCFv, 4) linker Ly, 5) the CD4 EXT and TM domains (residues 1–395 of the mature polypeptide), and 5) the ζ CYT domain (residues 31–142 of the mature polypeptide). These plasmids are constructed from three fragments: 1) a 6.7 kb vector fragment obtained by digesting pRT43.2F3 with EcoRI and ApaI, 2) a 2.2 kb fragment encoding the the entire 98.6-LLH SCFv domain, linker Lx, the entire 447D-LLH SCFv domain, linker Ly, and the N-terminal portion of the CD4 EXT domain, obtained by digestion of pIK-SAb (αgp41)-Lx-SAb(αgp120)-Ly-CD4-Fc with EcoRI and NheI, and 3) a 1.2 kb fragment encoding the C-terminal portion of the CD4 EXT domain, the CD4 TM domain and the ζ CYT domain, obtained by digestion of pIK43.2F3 with NheI and ApaI.

Example 8

Expression & characterization of MSCRs

To determine whether each MSCR polypeptide can be efficiently expressed and transported to the cell surface, and thus properly folded, a corresponding mammalian transduction-expression vector is used to transfect human 293 embryonic kidney cell line. Following transfection, the expression of each construct is evaluated by radioimmunoprecipitation, and its transport to the cell surface is evaluated by fluorescent-activated cell sorting (FACS) analysis.

Example 8A

Transfection of human 293 cells with NSCR expression vectors

For transfection, 293 cells were grown in DMEM:F12 media (JRH Biosciences) containing 10% fetal calf serum, and passaged at a 110 split ratio every 3 days. Twenty-four hours prior to transfection, 293 cells were plated at 5×10^5 cells per 10 cm culture dish. Ten μg of each expression plasmid DNA is transfected onto a 10 cm dish by the calcium phosphate coprecipitation method (Wigler et al. (1979) Cell 16:777). Twenty-four hours after transfection, the transfected cells were fed with fresh complete DMEM media. The expression of MSCR polypeptides was evaluated by FACS analysis and radioimmunoprecipitation analysis at 48 hours post-transfection.

Example 8B

FACS analysis of MSCR expression on 293 cells

Transfected 293 cells are rinsed once with PBS and incubated in PBS containing 10 mM EDTA for 5 minutes at room temperature. Cells are collected from plates, centrifuged and resuspended in PBS containing 1% fetal calf serum. Approximately 1×10^6 cells/sample are stained directly with saturating concentrations of FITC-conjugated mouse anti-human IgG or mouse anti-CD4 MAbs (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). Mouse FITC-IgG1 and PE-IgG2a are used as negative control MAbs. All FACS analyses are performed in a FACStation (Becton Dickinson) as previously described (Weiss and Stobo, (1984) *J. Exp. Med.,* 160:1284–1299).

Example 8C

Radioimmunoprecipitation of MSCRs expressed in 293 cells

Transfected 293 cells are rinsed once with RPMI medium lacking methionine. Cells are cultured for additional 8 hours in 2 M1 of methionine-deficient RPMI supplemented with 200 μCi [35S]-methionine (1160 C/mmol, ICN Biomedicals, Inc., Irvine, Calif.). The labelled cells are lysed in RIPA buffer, and the cell lysates are incubated at 4° C. for 1 hour with either no antibody (Class 1 MSCRs contain the IgG2 Fc domain and bind Protein A directly) or mouse anti-CD4 OKT4A MAb (Ortho Diagnostic Systems, Raritan, N.J.). Ten microliters of Pansorbin is added to the lysates to precipitate the MSCR. Immunoprecipitates are washed three times in RIPA buffer, boiled in SDS sample buffer and analyzed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels are fixed in 20% methanol/10% acetic acid, then soaked in Enlightning solution for 15 min, dried and subjected to autoradiography. SDS-PAGE analysis reveals the molecular mass of MSCRs expressed in 293 cells.

Example 9

Biochemical and biological properties of human CD8+ T cells expressing MSCRs

Example 9A

Infection of human CD8+ T cells with MSCR-expressing retroviral vectors

Human CD8+ T lymphocytes are isolated from peripheral blood lymphocytes (PBL) obtained from healthy donors by purification with the CEPRATE LC system (CellPro, Inc., Bothell, Wash.), followed by negative selection against CD4 cells using a T-25 MicroCELLector (AIS, Inc., Santa Clara, Calif.). Immediately after purification, cells are stimulated for 24 hours with an equal number of y-irradiated autologous PBMCs in AIM-V media (GibcoBRL, Grand Island, N.Y.) containing 10 ng/ml of OKT3 MAb and 100 units of human IL-2 (Chiron Corp., Emeryville, Calif.). Cells are then washed free of OKT3 and cultured in AR media (50% AIM-V, 50% RPMI, 4 mM Glutamine, 20 mM Hepes, 1 mM Na-Pyruvate, non-essential amino acids, and 100 units human IL-2) supplemented with 5% heat inactivated human AB plasma (Sigma, St. Louis, Mo.). Retrovirus is prepared in the TIN-4 cell line derived from thymidine kinase-expressing human 293 cells. For the transduction of human CD8+ +cells, TIN-4 cells are seeded at 5×10^5 cell/plate in 6-well plates (Corning Glass, Corning, N.Y.) in complete DMEM medium 48 hours prior to transfection. Ten micrograms of MSCEFR construct in the retroviral vector pRT43.2 (as described in U.S. patent application Ser. No. 08/258,152) are transfected per plate in the absence or presence of packaging plasmids by the calcium phosphate coprecipitation method. Following transfection, 1.5 M1 of fresh AR medium containing 100 units/ml of human IL-2 is added to each well of the plate. Three hours later, 5×10^5 of CD8+ T cells in AR media containing 100 units/ml of human IL-2 and 2 μg/ml of polybrene are added to each well of the plate. CD8+ T cells are removed from the 6-well plates 24 hours later and then transduced a second time by the same procedure. Newly transduced CD8+ T cells are maintained in AR media.

Example 9B

FACS analysis of MSCR expression in human CD8+ T cells

At various times following transduction, CD8+ T cells are harvested and washed with PBS containing 1% FCS. Approximately 1×10^6 CD8+ T cells are stained with specific antibodies for FACS analysis as described in Example 8B.

Example 9C

Immunprecipitation analysis of MSCR expression in human CD8+ T cells

At various times following transduction, human CD8+ T cells are harvested and placed in methionine-depleted AR media supplemented with 200 μCi [35S]-methionine (1160 Ci/mmol, ICN Biomedicals, Inc.). Cells are lysed in RIPA buffer and then incubated at 4° C. for 1 hour with either with no antibody (Fc-containing MSCRs) or mouse anti-CD4 OKT4A MAb (CD4-containing MSCRs). Ten microliters of Pansorbin are then added to the lysates to precipitate the MSCR polypeptide. The immunoprecipitates are washed three times in RIPA buffer, boiled in SDS sample buffer and analyzed by 8% SDS-polyacrylamide gel electrophoresis. Gels are fixed in 20% methanol/10% acetic acid, then soaked in Enlightning solution for 15 minutes, dried and subjected to autoradiography. SDS-PAGE analysis reveals the molecular mass of MSCRs expressed in human CD8+ T cells.

Example 9D

Cytolytic activity of MSCR-expressing human CD8+ T cells

To determine the cytolytic activity of MSCR-expressing human CD8+ T cells, in vitro cytolytic assays are carried out with target cells expressing wild-type and mutant HIV-1 antigens. HIV-1 infected human T cells or gp160-expressing 293 cells are compared with uninfected human T cells or untransfected 293 cells for their ability to be cytolytic targets for MSCR-expressing CD8+ T cells. Plasmids pIKenv+/rev+/tat−, pIKenvG370R/rev+/tat− and pIKenvR315Q/rev+/tat− are used to generate stably transfected 293 cells as described in U.S. Pat. No. 5,359,046, which express wild-type or mutant env proteins. These gp160-expressing 293 cells or HIV-1 infected human T cells are labeled at 37° C. for 18 hours with [^{3}H]TdR (Roberts et al, *Blood* 84:2878–2889 (1994)), washed and aliquoted to 96-well V-bottom plates at $\times10^4$/well. Serial dilutions of MSCR-expressing human CD8+ T cells are made up to achieve an effector to target (E:T) ratio ranging from 100:1 to 0.1:1. Samples are set up in triplicate and incubations are carried out for 6 hours at 37° C. Following incubation, aliquots of the culture supernatant are removed and counted in a liquid scintillation counter. Spontaneous release (SR) is obtained in a negative control sample lacking MSCR-expressing human CD8+ T cells; maximum release (MR) is obtained from a positive control sample by lysing target cells with 1N HCl. The percent specific lysis is calculated from the following equation:

$$\%\ \text{specific lysis}=(SR_{cpm}-\text{Sample}_{cpm})/(\text{Sample}_{cpm}-MR_{cpm})\times100\%.$$

The cytolytic activity of CD8+ T cells expressing various MSCRs and monospecific chimeric receptors as effector cells on target cells infected with various HIV-1 isolates or target cells transfected with wild-type or mutant env genes are compared. In particular, MSCRs which direct the efficient cytolysis of a range of primary HIV-1 isolates are considered good candidates for therapeutic application.

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 26

-continued

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGCGTCAAC ACAGACTGTG AGGAGACGGT GACCAG 36

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ACAGACTGTG AGCAGA 16

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTGCGTCAAC ACAGACTGAC CCTTACCCTC AGAAGATTTA CCCGACCCCG AGGTCGACCC 60

TGAGGAGACG GTGACCAG 78

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGAAGATTTA CCCGAC 16

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 111 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTGCGTCAAC ACAGACTGAC CGTCCTTCTT AGCGTCGTCC TTCTTAGCGT CGTCCTTCTT 60

AGCAGCGTCC TTCTTAGCGT CGTCAGCGGA AGATGAGGAG ACGGTGACCA G 111

-continued

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGTCGTCCT TCTTAG 16

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTGCGTCAAC ACAGACTGTG GGGACGGTGG GGATGTGTGA GTTTTGTCTG AGGAGACGGT 60

GACCAG 66

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGGTGGGGAT GTGTGA 16

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 87 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGCGTCAAC ACAGACTGGT CCAGCTCCCC GTCCTGCGCT TCGGCGCTCG ATTCTTCCAG 60

TTGCAGCTCT GAGGAGACGG TGACCAG 87

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCGGCGCTCG ATTCTT 16

-continued

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCCCAGCACC ACTTTCTTTC AGCTCACGGT GACCGT 36

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ACTTTCTTTG AGCTCA 16

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCCCAGCACC ACTTTCTTAC CCTTACCCTC AGAAGATTTA CCCGACCCCG AGGTCGACCC 60

TGAGCTCACG GTGACCGT 78

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 111 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCCCAGCACC ACTTTCTTAC CGTCCTTCTT AGCGTCGTCC TTCTTAGCGT CGTCCTTCTT 60

AGCAGCGTCC TTCTTAGCGT CGTCAGCGGA AGATGAGCTC ACGGTGACCG T 111

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCCCAGCACC ACTTTCTTTG GGGACGGTGG GGATGTGTGA GTTTTGTCTG AGCTCACGGT 60

GACCGT 66

-continued

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 87 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCCCAGCACC ACTTTCTTGT CCAGCTCCCC GTCCTGCGCT TCGGCGCTCG ATTCTTCCAG 60

TTGCAGCTCT GAGCTCACGG TGACCGT 87

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TAGTCTAGGA TCTACTGGCT GCAGTTCTTG CTCTCCTCTG TC 42

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ACTGGCTGCA GTTCTT 16

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AAAACTGTGC GTTACAATTC GTGGGTCCCC TCCTGAGGA 39

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TACAATTCGT GGGTCC 16

-continued

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TCCTATTGTA ACAAATGCTT GCCCTGGTCC TCTCTGGAT 39

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AAATGCTTGC CCTGGT 16

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Ser Ser Ala Asp Asp Ala Lys Lys Asp Ala Ala Lys Lys Asp Asp Ala
1 5 10 15

Lys Lys Asp Asp Ala Lys Lys Asp Gly
20 25

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Asp Lys Thr His Thr Ser Pro Pro Ser Pro
1 5 10

-continued

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Glu Leu Gln Leu Glu Glu Ser Ser Ala Glu Ala Gln Asp Gly Glu Leu
1 5 10 15

Asp

What is claimed is:

1. A DNA molecule encoding a chimeric membrane bound protein, said protein comprising in the N-terminal to C-terminal direction:

at least two extracellular inducer-responsive clustering domains in tandem to form a multispecific extracellular inducer-responsive clustering domain that binds specifically to at least one inducer molecule which results in the dimerization or oligomerization of said multispecific extracellular domain;

a transmembrane domain; and

a cytoplasmic signaling domain comprising a ζ or η chain of the T-cell receptor, or a Janus kinase,

wherein when said membrane bound protein is expressed in a selected host cell under conditions suitable for expression, said membrane bound protein initiates a signal in said host cell on binding to said at least one inducer molecule.

2. The DNA molecule according to claim 1 wherein at least one said extracellular inducer responsive clustering domain is an antibody or single-chain antibody or portions or modifications thereof containing inducer binding and clustering activity.

3. The DNA molecule according to claim 2 wherein at least one said extracellular inducer responsive clustering domain is a cell differentiation antigen.

4. The DNA molecule according to claim 1 wherein at least one said extracellular inducer-responsive clustering domain is CD4 and at least another said extracellular inducer-responsive clustering domain is an antibody or single chain antibody.

5. The DNA molecule according to claim 2 wherein at least one said antibody or single chain antibody recognizes an antigen from a virus selected from the group consisting of HIV, hepatitis A, B and C viruses, Kaposi's sarcoma associated Herpes virus, Herpes Simplex viruses, Herpes Zoster virus, cytomegalovirus, papilloma virus, respiratory syncytial virus and influenza virus.

6. The DNA molecule according to claim 2 wherein at least one said antibody or said single chain antibody recognizes an antigen on a cancer cell selected from the group consisting of interleukin 14 receptor, CD19, CD20, Lewis Y antigen, CEA, Tag72 antigen, EGF-R and HER-2.

7. The DNA molecule according to claim 1 wherein said transmembrane domain is naturally associated with one of said extracellular inducer responsive clustering domains.

8. The DNA molecule according to claim 1 wherein said transmembrane domain is naturally associated with said signaling domain.

9. A DNA molecule encoding a chimeric membrane bound protein, said chimeric protein comprising in the N-terminal to C-terminal direction:

at least two extracellular inducer-responsive clustering domains in tandem to form a multispecific extracellular inducer-responsive clustering domain that binds specifically to at least one inducer molecule which results in the dimerization or oligomerization of said multispecific extracellular domain;

a transmembrane domain;

a cytoplasmic domain comprising a Janus kinase; and

a cytoplasmic domain comprising ζ or η chain of the T-cell receptor;

wherein when said membrane bound protein is expressed in a selected host cell under conditions suitable for expression, said membrane bound protein initiates a signal for proliferation and effector function in said host cell on binding to said at least one inducer molecule.

10. A DNA molecule encoding a chimeric membrane bound protein, said chimeric protein comprising in the N-terminal to C-terminal direction:

at least two extracellular inducer-responsive clustering domains in tandem to form a multispecific extracellular inducer-responsive clustering domain that binds specifically to at least one inducer molecule which results in the dimerization or oligomerization of said multispecific extracellular domain;

a transmembrane domain;

a cytoplasmic domain comprising ζ or η chain of the T-cell receptor; and

a cytoplasmic domain comprising a Janus kinase;

wherein when said membrane bound protein is expressed in a selected host cell under conditions suitable for expression, said membrane bound protein initiates a signal for proliferation and effector function in said host cell on binding to said at least one inducer molecule.

11. The DNA molecule according to claim 9 or 10, wherein at least one said extracellular inducer responsive clustering domain is an antibody or single-chain antibody or portions or modifications thereof containing inducer binding and clustering activity.

12. The DNA molecule according to claim 9 or 10, wherein at least one said extracellular inducer responsive clustering domain is a cell differentiation antigen.

13. The DNA molecule according to claim 9 or 10, wherein at least one said extracellular inducer-responsive clustering domain is CD4 and at least one other said extracellular inducer-responsive clustering domain is a single chain antibody.

14. The DNA molecule according to claim 11, wherein at least one said single chain antibody recognizes an antigen from a virus selected from the group consisting of HIV, hepatitis A, B and C viruses, Kaposi's sarcoma associated Herpes virus, Herpes Simplex viruses, Herpes Zoster virus, cytomegalovirus, respiratory syncytial virus, influenza virus and papilloma virus.

15. The DNA molecule according to claim 9, wherein at least one said single chain antibody recognizes an antigen on a cancer cell selected from the group consisting of interleukin 14 receptor, CD19, CD20, Lewis Y antigen, CEA, Tag72 antigen, EGF-R and HER-2.

16. The DNA molecule according to claim 10, wherein said transmembrane domain is naturally associated with one of said multispecific extracellular inducer-responsive clustering domains, or ζ or η chain of the T-cell receptor.

17. The DNA molecule according to claim 9, wherein said transmembrane domain is naturally associated with one of said multispecific extracellular inducer-responsive clustering domains.

18. A DNA molecule encoding a hybrid extracellular and intracellular multispecific chimeric receptor protein, said chimeric protein comprising in the N-terminal to C-terminal direction:

at least two extracellular inducer-responsive clustering domains in tandem to form a multispecific extracellular inducer-responsive clustering domain that binds specifically to at least one inducer molecule which results in the dimerization or oligomerization of said multispecific extracellular domain;

a transmembrane domain:

a cytoplasmic domain comprising a Janus kinase; and

an intracellular inducer-responsive clustering domain comprising an immunophilin or a cyclophilin;

wherein when said chimeric receptor protein is expressed in a selected host cell under conditions suitable for expression, said receptor protein initiates a signal for proliferation in said host cell on binding to said at least one inducer molecule.

19. The DNA of claim 18, wherein at least one said extracellular clustering domains is an antibody or single-chain antibody or portions or modifications thereof containing inducer binding and clustering antibody.

20. The DNA of claim 18, wherein at least one said extracellular clustering domains is a cell differentiation antigen.

21. A DNA molecule encoding a hybrid multispecific chimeric receptor protein, said protein comprising in the N-terminal to C-terminal direction:

at least two extracellular inducer-responsive clustering domains in tandem to form a multispecific extracellular inducer-responsive clustering domain that binds specifically to at least one inducer molecule which results in the dimerization or oligomerization of said multispecific extracellular domain;

a transmembrane domain;

a cytoplasmic domain comprising a Janus kinase;

a cytoplasmic domain comprising a ζ or η chain of the T-cell receptor; and

an intracellular inducer-responsive clustering domain comprising an immunophilin or a cyclophilin;

wherein when said hybrid multispecific chimeric receptor is expressed in a selected host cell under conditions suitable for expression, said receptor protein initiates a signal for proliferation and effector function in said host cell on binding to said at least one inducer molecule.

22. The DNA of claim 21, wherein at least one said extracellular clustering domains is an antibody or single-chain antibody or portions or modifications thereof containing inducer binding and clustering activity.

23. The DNA of claim 21, wherein at least one said extracellular clustering domains is a cell differentiation antigen.

24. The DNA molecule according to claim 18 or 21, wherein said intracellular inducer responsive clustering domain binds to a natural or synthetic inducer that is cell membrane permeable and induces the clustering of said intracellular inducer responsive domain.

25. An expression cassette comprising a transcriptional initiation region, a DNA molecule according to claim 1 under the transcriptional control of said transcriptional initiation region and a transcriptional termination region.

26. An expression cassette comprising a transcriptional initiation region, a DNA molecule according to claim 9 under the transcriptional control of said transcriptional initiation region and a transcriptional termination region.

27. An expression cassette comprising a transcriptional initiation region, a DNA molecule according to claim 10 under the transcriptional control of said transcriptional initiation region and a transcriptional termination region.

28. An expression cassette comprising a transcriptional initiation region, a DNA molecule according to claim 18 under the transcriptional control of said transcriptional initiation region and a transcriptional termination region.

29. An expression cassette comprising a transcriptional initiation region, a DNA molecule according to claim 21 under the transcriptional control of said transcriptional initiation region and a transcriptional termination region.

30. The expression cassette according to claim 25, 26, 27, 28 or 29 wherein said transcriptional initiation region is functional in a mammalian host.

31. A cell comprising a DNA molecule according to claim 1.

32. A cell comprising a DNA molecule according to claim 9.

33. A cell comprising a DNA molecule according to claim 10.

34. A cell comprising a DNA molecule according to claim 18.

35. A cell comprising a DNA molecule according to claim 21.

36. A cell comprising an DNA molecule that encodes a chimeric effector function receptor comprising an extracellular inducer-responsive clustering domain, a transmembrane domain, and a effector function signaling domain and further comprising a second DNA molecule according to claim 1.

37. A cell comprising a DNA molecule that encodes a chimeric effector function receptor comprising an extracellular inducer-responsive clustering domain, a transmembrane domain, and a effector function signaling domain, and further comprising a second DNA molecule according to claim 9.

38. The cell comprising a DNA molecule that encodes a chimeric receptor comprising an extracellular inducer-responsive clustering domain, a transmembrane domain, a transmembrane domain and an effector function signaling domain, and a second DNA molecule according to claim 10.

39. A cell comprising a DNA molecule that encodes a chimeric effector function receptor comprising an extracellular inducer-responsive clustering domain, a transmembrane domain, and an effector function signaling domain, and further comprising a second DNA molecule according to claim **18**.

40. The cell comprising a DNA molecule that encodes a chimeric receptor comprising an extracellular inducer-responsive clustering domain, a transmembrane domain, a transmembrane domain and an effector function signaling domain, and a second DNA molecule according to claim **21**.

41. The cell according to claim **31, 32, 33, 34, 35, 36, 37** or **39**, wherein said cell is a mammalian cell.

42. The cell according to claim **31, 32, 33, 34, 35, 36, 37** or **39**, wherein said cell is a human cell.

43. A chimeric protein comprising in the N-terminal to C-terminal direction:

a multispecific extracellular inducer-responsive clustering domain comprising at least two domains in tandem that bind specifically to at least one inducer-molecule which results in the dimerization or oligomerization of said multispecific extracellular domain;

a transmembrane domain; and

a cytoplasmic domain comprising a ζ or η chain of the T-cell receptor, or a Janus kinase;

wherein when said chimeric protein is expressed as a membrane bound protein in a selected host cell under conditions suitable for expression, said membrane bound protein initiates a signal in said host cell on binding to said at least one inducer molecule.

44. A chimeric protein comprising in the N-terminal to C-terminal direction:

a multispecific extracellular inducer-responsive clustering domain comprising at least two domains in tandem that bind specifically to at least one inducer-molecule which results in the dimerization or oligomerization of said multispecific extracellular domain;

a transmembrane domain;

a cytoplasmic domain comprising a Janus kinase; and

a cytoplasmic domain comprising ζ or η;

wherein when said chimeric protein is expressed as a membrane bound protein in a selected host cell under conditions suitable for expression, said membrane bound protein initiates a signal for proliferation and effector function in said host cell on binding to said at least one inducer molecule.

45. A chimeric protein comprising in the N-terminal to C-terminal direction:

a multispecific extracellular inducer-responsive clustering domain comprising at least two domains in tandem that bind specifically to at least one inducer-molecule which results in the dimerization or oligomerization of said multispecific extracellular domain;

a transmembrane domain;

a cytoplasmic domain comprising ζ or η chain of the T-cell receptor; and

a cytoplasmic domain comprising a Janus kinase;

wherein when said chimeric protein is expressed as a membrane bound protein in a selected host cell under conditions suitable for expression, said membrane bound protein initiates a signal for proliferation and effector function in said host cell on binding to said at least one inducer molecule.

46. A chimeric hybrid binding protein comprising in the N-terminal to C-terminal direction:

a multispecific extracellular inducer-responsive clustering domain comprising at least two domains in tandem that bind specifically to at least one inducer molecule which results in the dimerization or oligomerization of said multispecific extracellular domain;

a transmembrane domain;

a cytoplasmic domain comprising a Janus kinase; and

an intracellular inducer-responsive clustering domain comprising an immunophilin or a cyclophilin;

wherein when said chimeric hybrid binding protein is expressed as a protein receptor in a selected host cell under conditions suitable for expression, said protein receptor initiates a signal for proliferation in said host cell on binding to either said inducer molecule or combinations thereof.

47. A chimeric hybrid binding protein comprising in the N-terminal to C-terminal direction:

a multispecific extracellular inducer-responsive clustering domain comprising at least two domains in tandem that bind specifically to at least one inducer molecule which results in the dimerization or oligomerization of said multispecific extracellular domain;

a transmembrane domain;

a cytoplasmic domain comprising a Janus kinase;

a cytoplasmic domain comprising ζ or η chain of the T-cell receptor; and

an intracellular inducer-responsive clustering domain comprising an immunophilin or a cyclophilin;

wherein when said chimeric hybrid binding protein is expressed as a protein receptor in a selected host cell under conditions suitable for expression, said protein receptor initiates a signal for proliferation in said host cell on binding to either said inducer molecule or combinations thereof.

* * * * *